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THERAPEUTIC POTENTIAL OF NATURAL KILLER CELLS IN MULTIPLE MYELOMA

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“I have not failed. I've just found 10,000 ways that won't work.”
Thomas Alva Edison

To my beloved wife and my daughter
“I go to sleep to dream because you are my dream.
Now, that I have you, my life needs no rest.”

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Abstract

Multiple Myeloma (MM) is an incurable plasma cell neoplasm with a median length of survival after diagnosis of approximately three years; therefore new treatment modalities to eradicate the disease are needed. Although the disease remains incurable, current main alternatives are drug treatment modalities and autologous stem cell transplantation (ASCT).

In order to study the contribution of autologous infused cells to relapse as well as the long term persistence of a transgene in haematopoietic cells following ASCT for MM, we genetically marked autologous $CD34^+$ enriched bone marrow or peripheral blood cell grafts from eight myeloma patients using retroviral vectors (paper I). The transgene could be detected for up to five years post-transplant in normal bone marrow cells, even in remission following relapse. No side effects related to retroviral gene transfer were observed. There were no marked myeloma cells observed in the patients either in remission or in relapsing disease. This supports the idea that lack of complete eradication of residual myeloma cells by conditioning, rather than the re-introduced myeloma cells during ASCT, is the cause of relapse.

Based on this finding, we set up a model to analyse new myeloma treatment modalities (paper II). In this study, 5T33MM cells were transduced with a retroviral vector coding GFP and injected to syngeneic C57BL/KaLwRij mice. Marked MM cells were successfully detected in different organs during disease development. The establishment of this model not only simplified the analysis of homing pattern of MM cells, but also eased the evaluation of therapeutic effects of different treatment approaches.

Using the C57BL/KaLwRij model, we determined anti-MM activity by NK cells following IL-2 administration, and if *ex vivo* activated and administered NK cell prolonged survival (paper III). Our data strongly support that IL-2 activated NK cells are not only the main effectors responsible for autologous myeloma cell killing in the C57BL/KaLwRij myeloma model, but also they increase life expectancy through adoptive transfer.

We are currently investigating the feasibility of expanding NK cells from MM patients with the aim of using them as a supportive or pre-emptive therapy. For this purpose, NK cells of 7 patients with MM were expanded in a clinical grade setting and their cytotoxic activity against autologous myeloma cells was evaluated. Our preliminary data show that *ex vivo* expanded primary human NK cells show autologous anti-myeloma activity.

Due to the effects of IL-2 on different cell populations *in vivo*, and the side effects such as cytokine leak syndrome in humans, we have created a retroviral vector that allows NK cells to be autoactivated by internal IL-2 production (paper IV). We then transduced an IL-2 dependent NK cell line as a proof of principle, and IL-2 dependent cells kept proliferating.

The above findings suggest that NK cells are important effector cells against MM and IL-2 is an important factor for their anti-myeloma activity. This indicates that IL-2 induced NK cells (gene modified or unmodified) can be analysed for feasibility in human settings.

List of publications

This thesis is based on the following publications. They are referred to in this thesis by their roman numerals.

I. Alici E, Björkstrand B, Treschow A, Aints A, Smith CIE, Gahrton G, Dilber MS Long Term Follow-Up of Gene Marked $CD34^{+}$ Cells after Autologous Stem Cell Transplantation For Multiple Myeloma. Accepted for publication in Cancer Gene Therapy (2006).

II. Alici E, Konstantinidis K.V, Aints A, Dilber M.S and Abedi-Valugerdi M (2004) Visualization of 5T33 myeloma cells in the C57BL/KaLwRij Mouse: establishment of a new syngeneic murine model of multiple myeloma. *Experimental Hematology,* **32**(11): 1064-72.

III. Alici E, Konstantinidis K.V, Sutlu T, Aints A, Gahrton G, Ljunggren H.G and Dilber M.S. Cytokine induced natural killer cells kill autologous myeloma cells in C57BL/KaLwRij. Manuscript.

IV. Konstantinidis K.V, **Alici E**, Aints A, Christensson B, Ljunggren H.G and Dilber M.S. (2005) Targeting IL-2 to the endoplasmic reticulum confines autocrine growth stimulation to NK-92 cells. *Experimental Hematology*, **33**(2): 159-164.

List of abbreviations

ADA	Adenosine deaminase
ASCT	Autologous stem cell transplantation
ATO	Arsenic trioxide
BM	Bone marrow
BMSC	Bone marrow stromal cells
CB	Cord blood
CC	Conventional chemotherapy
CD40L	CD40 ligand
CINK	Cytokine induced natural killer
CMV	Cytomegalovirus
CR	Complete remission
CTL	Cytotoxic T lymphocyte
DLI	Donor lymphocyte infusion
EBMT	Eurpoean group for blood and marrow transplantation
EBV	Epstein–Barr virus
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
GCV	Ganciclovir
GFP	Green fluorescent protein
GvHD	Graft versus host disease
GvL	Graft versus leukaemia
GvM	Graft versus myeloma
HDT	High-dose therapy
HHV8	Human herpes virus 8
HLA	Human leukocyte antigen
HS	Human serum
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
HSVtk	Herpes simplex virus thymidine kinase
i.p.	Intraperitoneal
i.v.	Intravenous
Id	Idiotype
IFN-γ	Interferon gamma
IL-	Interleukin
IL-2ER	Interleukin 2 endoplasmic reticulum retained expression
IL-2WT	Interleukin 2 wild type secreted form
IRES	Internal ribosome entry site
IU	International unit
KIRs	Killer Ig-like Receptors
KLH	Keyhold limpet hemocyanin
LAK	Lymphokine activated killer cells
LGL	Large granular lymphocyte
mAb	Monoclonal antibodies
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MM	Multiple myeloma
MOI	Multiplicity of infection
MoMLV	Moloney murine leukaemia virus
MPC	Mouse plasmacytoma
NCRs	Natural cytotoxicity receptors
neoR	Neomycin phosphotransferase II
NK	Natural Killer cells
NKT	Natural killer-like T cells
OCL	Osteoclast
OKT-3	Orthoclone monoclonal antibody against CD3
PBMC	Peripheral blood mononuclear cells
PBPC	Peripheral blood progenitor cells
PBS	Phosphate buffered saline
s.c.	Subcutaneous
SAP	Signaling lymphocyte activation molecule-associated protein
SBT	Sleeping beauty transposon
SCID	Severe combined immunodeficiency
SCT	Stem cell transplantation
TAA	Tumor associated antigen
TCR	T cell receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cells
VAD	vincristine, doxorubicin (Adriamycin), and dexamethasone
VEGF	Vascular endothelial growth factor

Introduction

Multiple Myeloma

Brief history

"Saturday, Nov. 1st 1845,

Dear Dr Jones,

The tube contains urine of very high specific gravity. When boiled it becomes slightly opaque. On the addition of nitric acid, it effervesces, assumes a reddish hue, and becomes quite clear; but as it cools, assumes the consistence and appearance which you see. Heat reliquifies it. What is it?"

The above quotation marks the first described case of multiple myeloma (MM) and was sent, along with a urine sample, by the London consultant physician Dr William Macintyre to the distinguished chemical pathologist Dr Henry Bence Jones[1-3]. The patient who had contributed the urine, Mr Thomas Alexander McBean, was later described by Macintyre[1] and had a history of progressive pain in "chest, back and loins", fatigue, and urinary frequency, with duration of about one year. The studies of Dr Bence Jones on the urine sample identified the contents as an oxide of albumin, and he also calculated that the patient had a daily excretion equivalent to 67 g of this substance. Mr McBean died in January 1846, and the autopsy revealed that the ribs, sternum and vertebrae were soft and brittle, and were filled with a soft, gelatinous matter. The microscopic examination demonstrated that this substance mainly consisted of round or oval cells with one or two nuclei, and drawings show that the appearance of these cells is consistent with what we now know as myeloma cells. Accordingly, this condition with softening of bones was designated "mollities ossium".

The term "multiple myeloma" was introduced in 1873. The classical full illustration of this clinical entity was first provided by Kahler in 1889, by describing the case of a fellow physician[4]. In 1928, a thorough emphasis on this description of the disease was done using all cases reported up to that time[5]. In 1917, the protein described by Bence wJones was reported to occur concomitantly in both serum and urine of patients[6], and

eleven years later, it was described that serum protein levels in myeloma patients could actually be elevated[7]. In 1939, myeloma serum globulins were separated by electrophoresis and the tall, narrow peak, now designated as the M-component, was described[8]. The investigation started by Bence Jones in 1845 was concluded in 1962, when the chemical nature of his "oxide of albumin" was defined by demonstrating the molecular similarity between the Bence Jones protein and light chains prepared from normal gammaglobulins. Dr Macintyre's question was finally answered after a century[9].

Demographics

The incidence of multiple myeloma in Sweden is about 60 new cases per million inhabitants per year. The frequency is strongly influenced by age. More than 50 percent of the patients are over the age of 70 years at the time of diagnosis, while only about 15 percent are under 60 years[10]. The incidence rate of myeloma is significantly effected by the ethnic background and is highest among the black race, followed by Maoris, Hawaiians, Israeli Jews, northern Europeans, US and Canadian whites[11]. Lowest rates occur in the Middle East, Japan, and China[12]. Another point worth mentioning is that the incidence rate of myeloma is significantly higher in males than females among both the black and white population[13].

Aetiology and pathogenesis of MM

Environmental factors

There is mismatching evidence about the relationship between environmental factors and the occurrence rate of MM. It has been suggested that smoking is related to an increased risk of developing MM[14] whereas another study in Sweden has shown no existence of such a relationship[15]. Likewise, it has been reported that incidence of MM is extremely high in American radiologists, suggesting a link between MM incidence rate and radiation exposure[16] but no such relation was observed during the studies on nuclear bomb survivors in Japan[17,18]. It has also been suggested that exposure to benzene increases the frequency of MM[19], however, this observation was not supported by later observations[20]. A direct correlation between the incidence of rheumatoid arthritis and MM has been shown in several studies[21,22] which suggests a

possible connection between B-cell malignancies and autoimmune disorders possibly due to the dysregulations in the immune system. However, it is not clear if MM is the result or the cause in these cases.

Genetic factors

The fact that MM incidence rate is remarkably different between races and also the observation that these patterns are well preserved regardless of migration to other areas, suggest that susceptibility to MM is determined by genetic rather than environmental factors. A series of studies have shown that predisposition to MM might be inherited. A study on the national database of familial cancer in Sweden has shown that males having fathers with cancer have a relative risk of 3.86 to develop MM[23]. Likewise, substantial familial clustering of MM has been reported in several studies which have also observed inherited identical HLA haplotypes among the affected family members[24-27]. Moreover, a significant association of the HLA-Cw2 allele with occurrence of MM has been reported[28].

Cytogenetic characteristics

Chromosomally abnormal clones can be found in 30-40% of patients with newly diagnosed disease, in 60% of patients with relapsed disease and in up to 80% of patients with plasma cell leukaemia. Loss of chromosome 13q is the most frequent observed case in MM karyotypes. Especially with interphase fluorescent *in situ* hybridisation (FISH) technique, it has been shown that 13q deletions occur in 39-54% of newly diagnosed patients[29]. Unfortunately, this common chromosomal abnormality is linked to a poor prognosis[29].

The IgH locus at 14q32 has strong transcriptional activity in B cells and the translocation of an oncogene to this region may result in dysregulation of its expression[30]. Expectedly, many B-cell tumours, including MM have chromosomal translocations mediated by VDJ recombination errors which position an oncogene under the influence of a strong immunoglobulin enhancer in this region. Cytogenetic analysis of myeloma shows that most of the non-hyperdiploid cases have one of the five recurrent IgH translocations: 16% *11q13*, 3% *6p21*, 5% *16q23*, 2% *20q12* and

15% *4p16* which result in the dysregulation of specific genes, respectively CCND1, CCND3, MAF, MAFB and FGFR3[31]. The remaining hyperdiploid tumours have multiple trisomies involving chromosomes 3, 5, 7, 9, 11, 15, 19 and 21, and infrequently one of these five translocations[31]. Nearly half of MM tumours are hyperdiploid and they generally have a better prognosis than nonhyperdiploid (<48 or >75 chromosomes) tumors[32].

Molecular mechanisms of pathogenesis

The level of cyclin D1, D2 or D3 expression in all MM tumours is significantly higher than in normal BM plasma cells[33] possibly due to the translocations mentioned above. This makes the myeloma cells more sensitive to proliferative stimuli from the BM microenvironment, and specifically BM stromal cells that produce IL-6, insulin-like growth factor 1 and several other cytokines[34]. This results in selective proliferation of tumour cells that produce several osteolytic factors including RANK ligand and large amounts of MIP-1α as well as immunosuppressive factors such as IL-10. Approximately 70% of MM patients have elevated levels of MIP-1α in their BM plasma[35]. MIP-1α directly stimulates osteoclast (OCL) precursors to differentiate into bone resorbing OCL[36,37] and thus results in increased rate of bone destruction. Also, adhesive interactions between myeloma cells and BM stromal cells induce increased production of RANKL and IL-6 by stromal cells and by this way increase OCL formation[38]. Besides, myeloma cells have also been shown to produce DKK1 that inhibits the WNT pathway which is critical for osteoblast differentiation[39]. Altogether, these changes in the BM microenvironment lead to the development of a tumour that will cause irreversible damage to bones and induce formation of osteolytic lesions.

Clinical findings

The main clinical symptoms of the disease are related to the accumulation of malignant plasma cells and their secretion of paraproteins. In early stages, MM is often asymptomatic, and may only be signalled by the finding of a high erythrocyte sedimentation rate in a routine laboratory test panel. In more advanced disease, the most common clinical findings are bone pain, pathological fractures and hypercalcemia, due to bone destruction. Cytopenia due to the bone marrow (BM) infiltration of myeloma plasma cells is also frequently occurring. The occurrence of a

monoclonal immunoglobulin (M-component) in serum and/or light Ig chains in the urine, resulting from the sustained ability for Ig production of the pathological plasma cells, is an important diagnostic tool. An increased risk of infections due to granulocytopenia and deficiency of normal polyclonal Ig may also be seen. Other findings may include renal failure and peripheral neuropathy.

The Durie-Salmon criteria are generally used for staging the disease[40].

Table 1: Staging criteria for MM

Stage	Criteria
I	Haemoglobin >100g/l Serum calcium <2.6 mmol/l Bone X-ray shows normal bone structure or solitary bone plasmacytoma only M component concentration: IgG <50g/l IgA <30g/l Urine light chain M-component on electrophoresis <4g/24h
II	Fitting neither stage I or III
III	One or more of the following: Haemoglobin <85g/l Serum calcium >3 mmol/l Advanced lytic lesions M component concentration: IgG >70g/l IgA >50g/l Urine light chain M-component on electrophoresis >12g/24h
Sub-classification	
A	Relatively normal renal function (Serum creatinine <170μmol/l)
B	Abnormal renal function (Serum creatinine ≥170μmol/l)

Current treatments

Drug therapy

Induction therapy

Patients who are eligible for ASCT are first treated with a regimen that does not damage the haematopoietic stem cells. Alkylating agents are avoided because of the risk that they may interfere with the mobilization of stem cells. Therapy for 3 to 4 months with vincristine, doxorubicin (Adriamycin), and dexamethasone (VAD) has been the standard induction therapy in the past. The disadvantages of this regimen are

the need for an indwelling venous catheter and the risk of related infections as well as thrombotic events. Thalidomide-dexamethasone is yet another regimen applied and patients receiving thalidomide-dexamethasone should also be given warfarin or low-molecular-weight heparin to prevent deep venous thrombosis.

All patients with multiple myeloma and lytic lesions on roentgenograms or osteopenia should receive intravenous bisphosphonates.

Induction therapy for patients ineligible for transplantation

Age, poor performance status, coexisting medical conditions, or prior therapy with alkylating agents may make patients ineligible for ASCT. Thalidomide-dexamethasone or VAD can be used for these patients, however, although response rates are better with melphalan-prednisone, there is no survival benefit[41]. Chemotherapy is usually continued until the patient reaches a plateau state, which is defined as a stable monoclonal protein in the serum and urine and no evidence of progression of myeloma. Generally this means a year of therapy. If there is a need for rapid response, such as in patients with renal failure or large, painful lytic lesions, high-dose dexamethasone or thalidomide plus dexamethasone can be used.

Results of both preclinical and clinical studies suggest that neoplastic cells are more sensitive to inhibition of the proteasome function than normal cells. For example, MM cell lines have been shown to be more sensitive to apoptosis induced by proteasome inhibitors than peripheral blood mononuclear cells from healthy individuals[42]. Although the biologic basis for the enhanced susceptibility of myeloma cells to proteasome inhibitors has not been fully elucidated, several hypotheses have been proposed, such as the sensitivity to proteasome inhibitors due to high rate of proliferation or more efficient uptake and slower inactivation of proteasome inhibitors by myeloma cells[43].

Thalidomide and its analogs

The rebirth of thalidomide[44,45] into active medical therapy has been one of the most important developments in the past 40 years of myeloma research[46]. The attempts to study the mechanism of action of thalidomide in the treatment of MM have not resulted in knowledge of an exact mechanism, but have opened the door to discovering other pathways and the creation of other theories to the disease's pathophysiology[47-49].

Thalidomide has a direct anti-myeloma effect in addition to its ability to modulate integrins. Modulating integrins interrupt the interaction between the myeloma cell and the BM stroma, significantly reducing the supportive cytokine environment and rendering the myeloma cell vulnerable and sensitized to different chemotherapeutic agents, including steroids[50]. Thalidomide has been shown to be active in the management of MM, both as a single agent and in combination regimens. Newly diagnosed and relapsed/refractory patients both benefit from the drug's activity[50-52]. This makes thalidomide a critical agent to use for salvage. Single-agent thalidomide has produced response rates (≥50% paraprotein reduction) in up to 30% of patients with newly diagnosed and relapsed/refractory disease[50-52]. When steroids are added to therapy, this response rate is increased up to 77% in patients with newly diagnosed disease[50]. When combined with steroids, paraprotein reductions of at least 75% in either serum or urine M-protein is observed in 72% of patients with newly diagnosed disease, including complete remission (CR) in 16% of patients[50].

Although the data for the use of thalidomide in newly diagnosed patients appear promising, data maturation is awaited before its use becomes the standard[53]. One of the potential uses of immunomodulatory agents such as thalidomide is to complement some of the benefits of chemotherapy either at standard dosages or high dose followed by salvage stem cell transplant.

Lenalidomide is 50–100-times more potent than thalidomide in augmenting IL-2 and IFN-γ production, and 50–2000-times more potent in stimulating T-cell proliferation triggered via the T cell receptor (TCR) and, in addition, lenalidomide triggers dose-dependent decreased secretion of TNF-α, IL-1β, IL-6 and triggers increased secretion

of IL-10[54]. This agent seems not to be associated with the significant somnolence, constipation or neuropathy commonly encountered with thalidomide. The traditional side effects noted with the use of thalidomide do not seem to be an issue with revlimid. Instead it is BM suppressive and induces frequently neutropenia and thrombocytopenia.

CC-4047 (actimid) possesses antiangiogenic activity[55] and augments anti-tumour responses *in vivo* following autologous tumour cell vaccination in a murine colorectal cancer model[56]. Currently there is no evidence of mutagenic or clastogenic potential for CC-4047. The most serious non-haematologic toxicity was deep venous thrombosis[57]. Further development of the compound is ongoing.

Proteasome inhibitors

Bortezomib (N-pyrazine carbonyl-l-phenylalanine-l-leucine boronic acid; previously known as PS-341 or MLN-341), a boronic acid dipeptide, is a specific inhibitor of the proteasome pathway[58-60]. Bortezomib inhibits the proteasome pathway in a rapid and reversible manner by binding directly with the 20S proteasome complex and blocking its enzymatic activity. The proteasome pathway is important for the activation of NF-κB by regulating the degradation of the IκB[61,62]. Several effects of bortezomib, including the induction of apoptosis in the malignant plasma cell, appear to be mediated through inhibition of NF-κB. Bortezomib prevents the degradation of IκB and thereby inhibits NF-κB activation[61,62].

The exact position of bortezomib in the management of MM and its future development await studies utilizing the drug in combination with other agents as well as longer follow-up in both newly diagnosed and relapsed/refractory myeloma patients. Moreover, due to its toxicity profile, especially past the first few cycles of therapy, and the need to have the agent for maintaining the response achieved, its use in new tolerable dosages and schedules as well as in combination therapy, especially as a chemotherapeutic or biologic sensitizer, is explored.

Arsenicals

Arsenic trioxide (ATO) is an agent of recent interest due to its impressive activity in patients with acute promyelocytic leukemia[63,64]. In addition, ATO holds therapeutic promise in the treatment of MM. ATO may inhibit tumour angiogenesis through direct and indirect mechanisms[65]. ATO induces growth inhibition and apoptosis in MM cell lines and freshly isolated human MM cells, where exogenous IL-6 does not overcome arsenic-induced growth inhibition or apoptosis[66]. ATO also induces anti-tumour activity through immunologic mechanisms[67]. The exposure of human myeloma-like cell lines and freshly isolated MM cells to ATO resulted in increased killing mediated by LAK cells, possibly through the upregulation of the CD38/CD31 and CD11a/CD54 receptor–ligand systems, which increase recognition, adhesion and lysis[67].

Agents in early development

Anti-CD40 molecules

CD40 is a type I transmembrane protein that is expressed on cells with high proliferative potential, including haematopoietic progenitors, epithelial and endothelial cells, and all antigen-presenting cells (including dendritic cells, activated B-lymphocytes as well as activated monocytes)[68-70]. CD40 is expressed on several types of B-cell neoplasms including MM, non-Hodgkin's lymphoma and chronic lymphocytic leukemia[71]. The high prevalence of CD40 expression found on B-cell neoplasms and its apoptosis-inducing characteristics have led to the investigation of this antigen as a target for immunotherapy.

The CD40 ligand (CD40L) is preferentially expressed on activated T-cells. CD40/CD40L interactions appear to be critical for cellular activation signals during immune responses and tumour cell growth. Binding of CD40 to CD40L has been shown to regulate immunoglobulin class switching; proliferation of normal B-cells, monocytes and dendritic cells; T-cell-dependent antibody production; and activation of antigen-presenting cells[72]. There are several anti-CD40 molecules in development and MM is one of the targeted diseases.

Vascular endothelial growth factor & its inhibitors

Emerging evidence suggests that human vascular endothelial growth factor (VEGF) may play an important role in the pathogenesis of myeloma. VEGF has been shown to be secreted by both myeloma and bone marrow stromal cells (BMSC), and its secretion is upregulated by binding of myeloma cells to BMSC[73]. VEGF secretion by myeloma cells augments IL-6 secretion by BMSC. Myeloma cells express high-affinity VEGF receptor, VEGFR-1. VEGF triggers phosphorylation of VEGFR-1 resulting in downstream activation of the MEK/mitogen-activated protein kinase (MAPK) pathway, thus inducing proliferation of myeloma cells. VEGF induces migration of myeloma cells via a protein kinase C (PKC)-dependent pathway. Small-molecule VEGFR tyrosine kinase inhibitors including PTK787/ ZK222584, GW654652 and GW786034 block VEGF-induced tyrosine phosphorylation of the VEGFR-1, MEK/MAPK activation-dependent proliferation, as well as PKC activation-dependent migration of myeloma cells[74]. Several compounds that appear to be potent inhibitors of VEGFR-1, -2 and -3 tyrosine kinases are in clinical development for a variety of human cancers, including MM. These agents appear to be promising, especially since the side-effect profile and the unique mechanism of action will allow for its use in combination therapy with other agents.

Histone deacetylase inhibitors

Acetylation and deacetylation of nucleosome histones play an important role in the modulation of chromatin structure, chromatin function and in the regulation of gene expression. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are two opposing classes of enzymes that tightly control the equilibrium of histone acetylation. An imbalance in the equilibrium of histone acetylation has been associated with carcinogenesis and cancer progression. The nucleosome is a subunit of chromatin that is composed of approximately 150 base pairs of two superhelical turns of DNA wrapped around histone proteins. When DNA is replicated, it unwinds, splits, replicates and then winds back up around these core histones. In this process, it is the terminal lysine-rich tail of the histones that can be modified by deacetylation or acetylation that will regulate the transcriptional activity of the specific genes. The HDAC removes the terminal acetyl group from the lysine tail and restores the positive charge to the lysine residue and condenses the nucleosome structure. HAT adds the

terminal acetyl group back on, thereby changing the charge of the terminal lysine so that the DNA uncoils around the core histones. Disruption of HAT and HDAC activity has been found in many human cancers. HDAC inhibitors are able to activate differentiation, arrest the cell cycle in G1 and/or G2, and induce apoptosis in transformed or cancer cells. Attention is currently being drawn to molecular mechanisms involving HDACs. An induction of p21Waf/Cip1 and a suppression of angiogenic stimulating factors have been observed in tumour cells following exposure to HDAC inhibitors[75-77].

Stem cell transplantation

Haematopoietic stem cells (HSC) have the ability to self-renew and to differentiate into all lineages of the haematopoietic system. The mechanisms for HSC differentiation into the mature cells of the varying lineages are not yet fully understood due to the complexity and uniqueness of the process for every single lineage. However, considerable progress in understanding these mechanisms with regards to the role of cytokines and transcription factors throughout the process, has been achieved[78].

A functional feature of HSC is their ability to reconstitute the haematopoietic system of a myeloablated organism[79-81]. This is achieved by the inherent ability of the stem cells to home to their niche in the BM, to self-renew, and to differentiate into all lineages of the haematopoietic system.

Adult human HSC can currently be obtained from three sources. They can be directly aspirated from the BM where they normally reside, or can be mobilized into the peripheral blood using cytokines or adhesion molecules. These mobilized cells are called peripheral blood progenitor cells (PBPC) and are the most commonly used source of HSC for transplantation into patients after myeloablation due to the rapid haematological recovery, the relative ease of stem cell extraction from the peripheral blood of the donor and possibly lower tumour contamination[82,83]. Although tumour cell contamination is lower in PBPC harvests than in BM, the superiority of PBPC autologous transplantation with regards to the clinical outcome has not yet been demonstrated. Sensitive immunofluorescence studies or PCR based techniques have

demonstrated that virtually all PBPC harvests are contaminated with malignant cells. Although the prognostic significance of detecting malignant cells with such sensitive methods is still unknown, attempts to reduce tumour cell contamination of the grafts have been a great concern due to the risk of causing relapse with the reintroduction of tumour cells along with ASCT.

The third source of HSC is the usually discarded umbilical cord of newborn babies. Studies have shown that, unfractionated umbilical cord blood (CB) cells have the highest frequency of cells capable of reconstituting the haematopoietic system of immune compromised mice, with 3 times more repopulating cells than BM, and 6 times more than PBPC[84]. However, the low total stem cell number obtained from a single cord blood harvest, and the lack of banking systems, continues to limit their practical use in adult stem cell transplantations.

In the absence of significant improvement of conventional chemotherapy (CC), high-dose therapy (HDT) along with ASCT has been increasingly used in the past decades to treat MM[85]. Non-randomised studies have shown that, for patients responding to initial induction chemotherapy, ASCT is a safe with less than 5% transplant related mortality (TRM) and effective consolidation therapy[86,87].

Another treatment option, allogeneic stem cell transplantation (SCT) has two major drawbacks in MM. Firstly, since it is usually performed in patients under 50 years of age and having an HLA identical sibling, it can be proposed only to a small minority of MM patients. Secondly, the transplant related mortality in allogeneic SCT is very high, mainly as a consequence of either infection or Graft-Versus Host Disease (GvHD). Analysis of the Eurpoean group for blood and marrow transplantation (EBMT) data showed no advantage of allogeneic SCT compared to ASCT[88] yet, there are strong arguments in favour of pursuing allogeneic SCT in MM.

Studies have shown that, if given early in the course of the disease, allogeneic SCT yields molecular remissions and about one third of the patients remain free of disease 6 years later[89,90]. Due to the anti-tumour effect of the graft, allogeneic SCT is currently the only genuinely curative therapy in MM. Reports of CR achieved after infusion of donor mononuclear cells in patients relapsing after allogeneic SCT are

further evidence of the so-called graft versus myeloma effect[91-94]. Therefore the objective of current studies on allogeneic SCT in MM is to reduce transplantation related mortality while still harnessing graft versus myeloma effect.

The use of reduced intensity conditioning (RIC) non-myeloablative transplantation is an attractive alternative and may be an option for older patients. The use of non-myeloablative conditioning regimen using melphalan (100 mg/m^2) combined with donor lymphocyte infusions (DLI) has induced good disease control one year but with a significant rate of GvHD in patients with high-risk MM[95]. Since early transplant-related mortality is reduced with non-myeloablative conditioning regimen this approach is currently tested in front line treatment. However preliminary studies in various haematologic malignancies have clearly shown that results are related to disease status and that relapse rate is high in patients with advanced disease. Therefore attempts to reduce tumour burden with ASCT prior to RIC allotransplantation has been proposed. Although GvHD could remain a problem, feasibility of this approach has been demonstrated[96]. Preliminary results are encouraging but the follow-up is still short.

Although high-dose chemotherapy and total body irradiation prior transplantation effectively reduce the plasma cell burden, donor derived immunocompetent cells have been shown to play an important role in the curative effects of allogeneic HSCT[97]. DLI alone have been demonstrated to induce response rates in 40-67% of patients with MM[91,98-104]. The response rates in the two large trials on DLI for MM relapse after allo-HSCT were 22-28% CR and 14-30% PR[105,106]. Beside the relapse treatment of MM after HSCT, DLIs have been applied as part of the transplantation strategy after nonmyeloablative regimens. CD8$^+$ depletion in DLIs as consolidation treatment resulted in a response rate of 71%, with 43% CR[98].

Responses were mostly observed after relatively high T cell doses achieved by multiple DLIs. The response to DLI treatment was highly correlated with the occurrence and severity of acute GvHD in one study and in the other study no correlation was detectable[105,106].

Beside the immune escape of plasmacytoma in extramedullary tissues, infectious complications and graft failure[105,107], the clinically most relevant DLI related morbidity is the occurrence of GvHD[91,92,98-100,104,105,108]. In the two larger studies on DLI for myeloma, acute GvHD was documented in 52-56% and chronic GvHD in 26-44% of patients[105,106].

Acute GvHD is mediated primarily by donor T cells directed against multiple antigens expressed in different recipient tissues. Despite a potential overlap between GvHD and graft-versus leukaemia (GvL) effect, several animal models have demonstrated the feasibility of distinguishing these two effects of donor T cells[109-111]. Different clinical strategies to preserve GvL while reducing the incidence of GvHD after DLI include infusion of limited numbers of donor T cells, sequential infusion of increasing numbers of donor T cells and infusion of selected subsets of donor T cells[112]. $CD8^+$ T cell depletion of the DLIs prior infusion appears to be one method for effectively reducing the incidence and severity of GvHD[112].

Reduced-intensity transplantation, including *in vivo* T cell depletion with alemtuzumab and adjuvant dose-escalating DLIs showed very promising results [113]. DLI can be employed as a part of the treatment strategy for patients with persistent, relapsed or progressive disease after nonmyeloablative haematopoietic cell transplantation.

Despite the widespread use of DLI to treat or prevent relapse, the immunological mechanisms mediating graft versus myeloma (GvM) activity are not yet fully defined. The identification of target antigens present on MM cells and absent on non-malignant cells would be of major interest to induce MM-specific donor T cells *in vitro* for adoptive transfer and therefore improving the safety and efficiency of DLI therapy.

Experimental models of MM

To illuminate the nature as well as the immunobiology of MM, different *in vitro* and *in vivo* systems have been described. The existence of various human myeloma and BM stromal cell lines makes *in vitro* studies relatively easy[114-116]. However, experimental possibilities of *in vitro* systems are limited in general and important findings with these simplified *in vitro* systems need to be validated in an *in vivo* situation before the obtained knowledge can be applied to humans. Moreover, most of the human MM cell lines are derived from refractory cells and are therefore not representative for *in vivo* MM cells. The *in vivo* models provide a multidimensional network of interactions which overcome extrapolation problems of *in vitro* observations. In addition, *in vivo* models are necessary for preclinical studies. Therefore, good experimental models of MM are of utmost importance.

A good *in vivo* model of MM should of course, be easy to maintain, reproduce the characteristics of the human disease, produce similar clinical symptoms a well as laboratory abnormalities and behave in a similar way to the human disease in terms of the observed responses against basic MM treatment. Several models for the disease have been developed up to now, yet many of them do not completely fulfil the criteria mentioned above.

The pristane oil-induced mouse plasmacytoma (MPC) model is one of the most widely used models and has provided considerable data on plasmacytomagenesis[117]. However, MPC growth is usually restricted to the peritoneal environment and typical features of human MM such as bone lesions, anaemia and renal impairment have not been described in this model.

The important role of IL-6 in the pathogenesis of MM has led to development of IL-6 transgenic mouse models for clarifying its role[118]. These transgenic mice as well as studies with retroviral gene transfer facilitate the observation of oncogenes in neoplastic transformation and give information about the effect of specific genes in the pathogenesis of MM. Nevertheless, creation of transgenic mouse strains labourious and the retroviral gene transfer models have drawbacks due to the

coexistence of a mixed cell population of partially transgene expressing cells and normal cells.

A disseminated-like disease can be reproduced in severe combined immunodeficient (SCID) model[119,120] by inoculation with human primary MM cells or leukaemia cell lines. Multiple osteolytic bone lesions and BM involvement are generated in this model, and conventional drugs applied in the treatment of human multiple myeloma have proven to be effective[121]. Nevertheless, the immune system of SCID mice is totally different from that of a MM patient and the information gathered by this way does not exactly reflect the real situation.

Ageing mice of the inbred strain C57BL/KaLwRij develop monoclonal proliferative B-cell disorders with a high frequency. About 80% of these mice have monoclonal gammopathy of undetermined significance (MGUS), similar to that in humans. In a few cases (1%) mice older than two years develop MM and, with about the same frequency, Waldenström's macroglobulinaemia[122-124]. The bone marrow cells of these disease bearing mice during disease have previously been acquired and derived into various cell lines which are called the 5TMM series and have been extensively characterised[125]. 5T33MM models have been initiated by successful intravenous transplantation of primary MM cells into syngeneic recipients and have since been propagated by intravenous injection of BM cells from 5TMM diseased mice into young syngeneic animals[126]. In analogy to human MM, 5T33 MM cells are mainly localised in the BM (and in the spleen which is, in contrast to the human situation, a haematopoietic organ in the mouse). Tissue distribution of these cells also includes lymph nodes and thymus. Similar to the human situation, the recipients develop high levels of tumour-related monoclonal immunoglobulins in the serum, together with a decreased concentration of normal polyclonal immunoglobulins[127]. Another close resemblance with the human disease is the development of bone destruction in the animals. Radiographic and histological observations show the presence of several osteoporotic and osteolytic lesions in the bone, which are consistently observed in the 5T33MM model.

Characteristics of the 5T33MM models are similar to those of human MM[128]. Furthermore, the simple and reproducible successive continuation of the model in this

immunocompetent mouse strain makes it highly suitable as a good *in vivo* model for investigations of the pathobiology and for preclinical studies of human MM including studies of the role of immune system in MM and possible applications of immunotherapy.

Although the systemic nature of haematological malignancies may be recapitulated by *in vivo* models of i.v. injections of tumour cells such as in 5T33MM model, these models do not readily allow for sensitive detection of the exact anatomical sites of development of lesions or for comprehensive monitoring of their progression.

The immune system in MM

The development of cancer clearly indicates that the immune system of the patient has failed in the surveillance against malignant cells. The immune response is lacking ability to eliminate the tumour due to either tolerance or immunosupression[129,130]. Tumour associated antigens (TAA) which are, in most of the cases, "self proteins" inherently show no immunogenicity[131,132]. The notion that cancers are comprised of healthy cells and therefore do not send out "danger signals", as well as the immune tolerance to self TAAs suggests that tumours may simply be ignored by the immune system[133].

"Cancer immunotherapists… They are truly heroes. They believe that they can dry the ocean with a hair dryer." George Klein, ESGT 2005 meeting, Keynote speech

Illustration is a gift from Cem Dinlenmis.

In certain cases, where a robust anti-tumour immune response is generated, tumours may devise escape mechanisms to avoid destruction by the immune system. These mechanisms include down-regulation or loss of major histocompatibility complex (MHC) expression as well as the expression or secretion of certain immunosuppressive factors[134,135].

A significant impairment of T cell function is common in MM patients as well as patients with MGUS. Certain abnormalities in T cell repertoire, phenotype and function following high dose chemotherapy and autologous HSCT are also observed in MM patients[136-138]. In both untreated and conventionally treated MM patients, a

significant reduction in frequency and proliferative capacity of $CD8^+$ T cells against Epstein–Barr virus (EBV) and influenza A is observed[136]. $CD4^+$ T lymphocyte numbers, particularly the naïve $CD4^+/CD45RA^+$ subset, are also reduced in MM patients[139]. Downregulated expression of CD28 on expanded T cells of MM patients, which may impair priming, is also observed[137]. Both $CD4^+$ and $CD8^+$ T lymphocytes from MM patients exhibit a marked reduction in the expression of T-cell signalling and signal transduction molecules, as well as production of cytokines IFN-γ, IL-2 and IL-4[140]. Not surprisingly, these defects increase with disease progression[140]. Despite these abnormalities, T-lymphocyte expansions are found in MM patients and they have the phenotype of CTL[141]. In addition, the presence of expanded T cell clones with TCR Vβ gene rearrangements confers an improved prognosis, and shows a degree of correlation with the presence of Idiotype (Id) reactive T cells[142].

Although phenotypic and functional aberrations in CD4 and CD8 cells have been described in MM and MGUS[143-146], the biologic basis for these abnormalities remains unclear. A possibility is that the abnormal regulatory T cells (T_{reg}) cell activity in myeloma patients could contribute to immune dysfunction in MM and could provide a new target to enhance immune responses. T_{reg} cells are key players in the control of immune system, taking part in maintenance of self-tolerance, control of auto-immunity, and regulation of T cell homeostasis and thus they are modulators of immune responses against infections and malignancies[147].

Additionally, MM is reported to be associated with a number of autoimmune disorders, such as thyroid abnormalities[148], rheumatoid arthritis[149], and renal complications[150]. It is still not clear whether these conditions are the cause or the effect of dysfunctional T_{reg} cells in MM.

Immunotherapy in MM

Chemotherapy might disrupt TCR diversity and have a negative effect on T-cell function in MM patients[151]. Yet, vaccination with Id/keyhold limpet hemocyanin (KLH) conjugates can recover this loss in TCR diversity[152]. NKT cells are dysfunctional in MM patients with progressive disease, but it has been demonstrated that this can potentially be reversed using stimulation with α-galactosylceramide

pulsed DC[153]. Another important finding is that, even in patients with progressive disease, it is possible to generate cytotoxic T lymphocytes (CTL) specific for malignant plasma cells[154,155] and MM-associated TAAs[156-158] from the T cell precursor pool of the patients[153,154].

The presence of a high frequency of Id-specific B-cell[159] and T-cell responses *in vitro* has been observed[160-162] which apparently demonstrated the immunogenicity of Id. However, high levels of free Id have been correlated with deletion of anti-Id T cells in MM[163]. Thus, despite the promising potential, clinical studies targeting Id in MM have been disappointing. The inefficiency of this approach due to high Id levels indicates that using Id as a TAA for MM immunotherapy would be most suitable in the early stages, or during a minimal residual disease.

Anti-Id T and B cell responses were seen after repeated immunisations with autologous Id without clinical benefit[164]. Different adjuvant combinations for improving this immune response have been tried. In a study, all five patients vaccinated with combined G-CSF and Id showed anti-Id immune responses[165]. The second approach used to increase the immunogenicity of the Id protein was to conjugate it to the carrier/helper protein KLH. Here, the anti-Id responses were weak, although the patients were immunocompetent and showed strong responses to KLH[166]. Follow-up of these patients indicated no superiority to conventional relapse therapy with IFN-α alone or combined with steroids[152].

DCs have also been used in MM immunotherapy approaches for improving the effect of anti-Id vaccination due to their specialised capacity to capture and present antigens. The two published trials using autologous gradient-separated DCs pulsed with Id[167,168] present mildly encouraging results with strong responses to KLH, Id-specific proliferation as well as some indication of a fall in Id protein levels.

Natural killer cells

Historical background

Natural killer (NK) cells were initially discovered due to their ability to spontaneously kill certain tumour cells. The cells were discovered at the Karolinska Institutet by Kiessling and Klein, who also named these cells natural killer cells[169,170] and in parallel by Herberman and colleagues in the USA[171,172]. Human NK cells were described as nonadherent, nonphagocytic, $Fc\gamma R^+$ large granular lymphocytes (LGL)[173]. Later it was, however, appreciated that not only NK cells shared the LGL phenotype and that some NK cells displayed normal small lymphocyte morphology, depending on their activation status[174]. This made it difficult to separate the NK cell population just by the size and morphology. The identification of the NKR-P1[175], and NK1.1[176] made it possible to define the murine NK cell population roughly as $NK1.1^+$ TCR^- sIg^- $CD16^+$.

As the name implies, NK cells can kill certain cells without prior sensitization, but they are also potent producers of various cytokines, such as IFN-γ, TNF-α, GM-CSF and IL-3[177]. Therefore NK cells are also believed to function as regulatory cells in the immune system, influencing other cells and responses[178]. For example, NK cells participate in the development of the autoimmune disease, myasthenia gravis, by regulating both the autoreactive T and B cells through their production of IFN-γ[179].This is interesting because it shows a link between the adaptive and the innate immune systems. Other studies have shown a close interaction between NK cells and dendritic cells[180].

NK cells are also believed to be involved in the defence against virus infections. A patient lacking NK cells reportedly had severe forms of virus infections with various herpes viruses[181].

The discovery of NK cells suggested a possible effector mechanism behind the phenomenon of “hybrid resistance”. Skin and organ transplantations had shown that allogeneic grafts were rejected while syngeneic grafts were tolerated, i.e. rejection only took place when the grafts had MHC molecules differing from the host. This

rejection was mediated by T cells [182,183], which could induce either a graft-versus-host or a host-versus-graft reaction. Irradiated (AxB)F_1 mice rejected BM transplants from either parent, despite the fact that the transplant did not express any foreign MHC molecules. This was not in accordance of the reigning dogmas of T cell mediated rejection. The BM rejection could still be observed in SCID mice, which have no T and B cells but have functional NK cells[184].

Development of NK cells

The BM microenvironment provides a rich source of cytokines and growth factors that can support NK cell development *in vitro* and contains stromal cells that are required for full maturation of NK cells. Early studies showed that NK cell generation from human $CD34^+$ HSCs required stromal-cell contact, whereas more-differentiated $CD34^+CD7^+$ cells could generate NK cells when cultured in IL-2 alone[185]. Similarly, fetal liver $CD34^+CD38^+$ cells could generate NK cells *in vitro* using IL-15[186]. It was later reported that the stromal cell requirements could be replaced by early-acting cytokines such as stem cell factor also known as c-kit ligand, fetal liver kinase 2 ligand, also known as FMS-like tyrosine kinase 3 ligand and IL-7[187,188].

HSCs give rise to precursors of different haematopoietic lineages. The first step of HSC commitment generates earliest and then common lymphoid progenitors[189,190], which further differentiate to give rise to B, T and NK precursors. Human natural killer cell progenitors have not been fully characterized *ex vivo*; however, the first IL-2Rβ-expressing cells that emerge following culture of human $CD34^+$ HSCs with C-kit ligand or FLT3L have been shown to express CD38 and transcripts for IL-15R, and are negative for CD7, CD16, CD56 and several NK cell receptors[191]. During this process NK cells establish a characteristic cell surface phenotype and the capacity to elicit effector functions. Careful phenotypic and functional analyses of rare immature NK cells isolated from fetal liver, cord blood and BM, or those that develop *in vitro* from HSCs support a linear model of NK cell maturation[185,192,193].

Biology and function of NK cells

NK cells have been described as "large granular lymphocytes" and the granularity of NK cells is one of the keys to how they kill target cells. The granules contain perforin and granzyme B[194]. It was initially believed that polyperforin lesions disrupt the target cell membrane but there is no evidence for such an activity yet. Besides, granzyme uptake is perforin independent and is done through receptor mediated endocytosis. It is postulated that granzymes and perforin both bind to the target surface as part of a single macromolecular complex[195]. When an NK cell encounters a target cell, perforin and granzyme B are released; granzyme enters the target cell and mediates apoptosis while perforin disrupts endosomal trafficking[196,197]. Activated NK cells can express FasL, which induces apoptosis by binding Fas on target cells[198]. FasL is a member of the TNF family and another member, TRAIL, is also expressed by activated NK cells. TRAIL can bind to receptors on the target cells[199]. TNF-α has also been suggested to mediate activation-induced cell death by NK cells[200].

Cytokines and NK cells

IL-2 is a multifunctional cytokine considered to be a NK cell proliferating and activating factor [201]. In B cells it stimulates growth and the synthesis of the J-chain and in T cells it stimulates growth -in particular inducing a large number of $CD4^+$ T cells[202]. Among other haematopoietic cells it stimulates NK cell growth, and induces lymphocyte and NK activity[203]. The clinical interest for IL-2 and its receptor is based on the fact that it has a central role for the immune response. Activated T cells, in the presence of a foreign molecule, release IL-2.

The receptor components of the IL-2 family of cytokines are type I cytokine receptors (haematopoietic receptors)[204]. IL-2R can, due to its structural motif, share a receptor component with other cytokines[205,206]. This receptor component is γ_c and is shared by IL-2R, IL-4R, IL-7R, IL-7R and IL-15R. The basis of the fact that γ_c can be shared is that it is a component that can be utilized differentially from one receptor to another depending on the presence of different ligands. However sharing receptor components, like in the case of γ_c, may provide a mechanism through which different

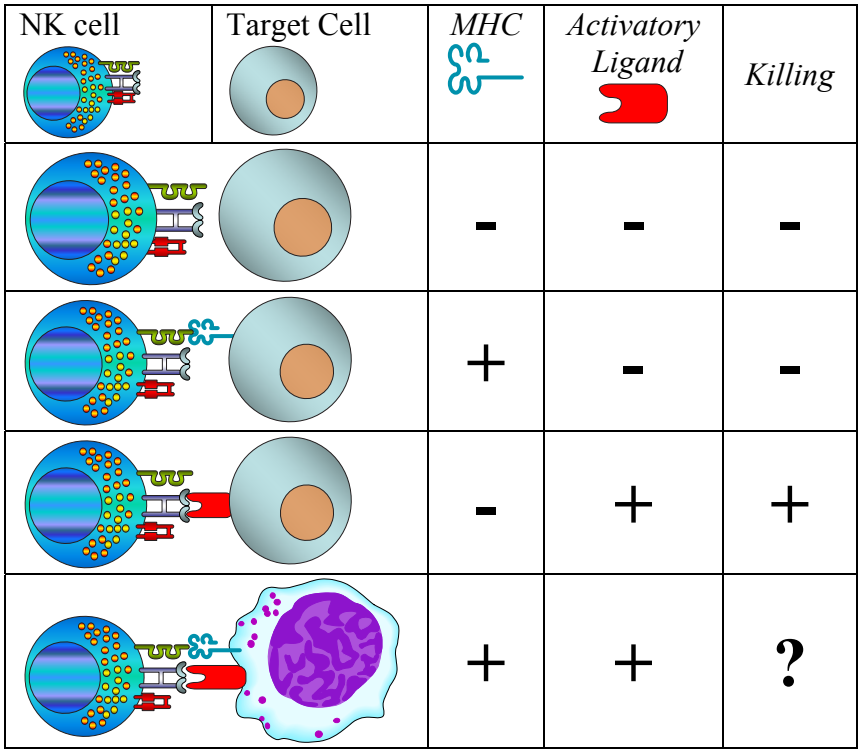
cytokines control each other's activities and can allow for the sharing of actions. IL-2 was the first cytokine to be identified and cloned almost 25 years ago[207].

Other interleukins such as IL-12, IL-15, IL-18 and IL-21 have also been suggested to contribute to NK cell function[208-212]. In particular IL-12 activates NK cells that secrete IFN-γ, switches T cells to T helper cells (Th_1) and inhibits IL-4-induced IgE secretion. IL-12 was initially discovered due to its ability to stimulate interferon-γ production by NK cells and to enhance CD8 cytotoxicity. Similarly, IL-15 induces growth and cytotoxicity of NK cells and it leads to NK cell differentiation. The combination of IL-15 and IL-21 enhances the production of IFN-γ in human NK cells, a fact that can also be achieved from the synergetic effect of IL-12 and IL-18 as well.

The Missing self concept

Initially it was not clear how NK cells distinguished target cells they should kill from those that they should spare. When Kärre summarized his and other peoples work in his thesis, he found a common denominator when considering what was missing on the killed targets cells instead of trying to find something that was commonly expressed. This lead him to formulate the "missing self" hypothesis, where he suggested that NK cells kill target cells lacking expression of self MHC class I molecules although the mechanism was very unclear[213,214]. The effector inhibition model was then shown by the discovery of inhibitory receptors on NK cells some years later[214]. Missing self could also explain the hybrid resistance phenomenon, since the $(AxB)F_1$ host killed cells from either parent A or B because these cells lacked complete self MHC expression (A+B). To test the missing self-hypothesis, a MHC class I-deficient version of the tumour cell RMA ($H\text{-}2^b$) was established and was named RMA-S. C57BL/6 mice inoculated with RMA-S cells rejected the tumours, while mice inoculated with RMA developed the tumour. By treating the mice with NK depleting anti-Asialo GM1, the difference in tumour outgrowth disappeared[215].

Figure 1: The missing self concept



NK inhibitory and activating receptors

NK cytotoxicity is the result of a balance between the inhibitory and activatory receptors. Upon recognition of the ligands on the target cell surface by NK receptors, various intracellular signalling pathways drive NK cells towards cytotoxic action and this results in target cell cytolysis[216].

However, these processes are tightly controlled by a group of inhibitory receptors. These receptors act as negative regulators of NK cytotoxicity and inhibit the action of NK cells against "self" targets. A main group of this type of receptors is the Killer Ig-like Receptors (KIRs) which are mainly specific for MHC Class-I molecules. If the target cell is recognized by KIRs, which means, it has sufficient amount of self MHC Class-I molecules on the cell surface, an inhibitory signal from KIRs stops the action of cytotoxic pathways triggered by activating receptors. The KIRs are type I (extracellular amino terminus) membrane proteins that contain either two or three extracellular Ig-like domains[217] and they are designated KIR2D or KIR3D, respectively. The cytoplasmic domains of the KIRs can be either short (S) or long (L),

corresponding to their function as either activating or inhibitory receptors, respectively. Members of the KIR family recognize HLA-A, HLA-B and HLA-C alleles, and KIR2DL4 recognizes HLA-G[218]. This provides that even the loss of a single HLA allele (a common event in tumorigenesis and viral infection) can be detected by a pool of NK cells[219].

Table 2: List of adhesion, activating and inhibitory receptors identified on NK cells

Receptor	Sp	Class	Motif / Adaptor	Ligand	Ref
Adhesion Receptors					
CD2 (LFA-2)	H,M	IgSF	Proline-rich domain	CD48, CD58 (LFA-3)	220
CD11a (LFA-1)	H,M	IgSF	Src family kinases, PI3K	CD54 (ICAM-1), CD102 (ICAM-2)	221
CD11b (Mac-1)	H,M	IgSF	?	CD54 (ICAM-1)	221
CD43 (sialoadhesin)	H,M	IgSF	?	?	222
CD44	H,M	IgSF	?	Hyaluronic acid	223
CD49b (DX5)	M	IgSF	?	?	224
CD56 (N-CAM)	H	IgSF	?	?	225
CD223 (Lag3)	H,M	IgSF	?	HLA Class II	226
Activating Receptors					
CD16 (FcγRIII)	H,M	IgSF	ITAM / FcγR	Immune complexes	227
CD25 (IL-2Rα)	H	CytoR	?	IL-2	228
CD27	H,M	TNFRSF	TRAF	CD70	229
CD28	H,M	IgSF	YXXM / PI3K	CD80, CD86	230
CD69	H,M	C-lectin	?	?	231
CD94/NKG2C,E	H,M	C-lectin	ITAM / DAP12	HLA-E in human, Qa-1b in mouse	232
CD122 (IL-2Rβ)	H,M	CytoR	JAK1,3 / STAT5a,b	IL-2, IL-15	233
CD161	H,M	C-lectin	?	Clr-g (NKR-P1F)	234
CD226 (DNAM-1)	H	IgSF	?	CD112 (Nectin-2), CD155	235
CD244 (2B4)	H,M	SLAM	TXYXXV-I / SAP,Fyn	CD48	236
CD314 (NKG2D)	H,M	C-lectin	YINM / DAP10, PI3K	MICA, MICB, ULBs, RaeIs, H60	237
KIR2S, KIR3S	H	IgSF	ITAM / DAP12	HLA Class I	238,239
Ly49D, H, P	M	C-lectin	ITAM / DAP12	H-2 Class I, MCMV m157 (Ly49H)	240
NCRs (NKp30, 44, 46)	H,M	IgSF	ITAM / FcγRIII, CD3ζ, DAP12	Viral hemagglutinins (?)	241-243
CD85H (ILT-1)	H	IgSF	ITAM / FcγR, DAP12	?	244
IFN-α/βR	H,M	CytoR	JAK1, Tyk2 / STAT1, 4	Type I interferons	245
gp49A	M	C-lectin	ITAM	?	246
Inhibitory Receptors					
CD85 (ILT-2)	H	IgSF	ITIM / SHP-1	HLA-A, -B, -G	247
CD94/NKG2A	H,M	C-lectin	ITIM / SHP-1, -2	HLA-E in human, Qa-1b in mouse	248
CD161	M	C-lectin	ITIM / SHP-1, -2	Clr-b (NKR-P1D)	234
CD244 (2B4)	H,M	SLAM	TXYXXV-I / SAP,Fyn	CD48	236
KIR2DL, KIR3DL	H	IgSF	ITIM / SHP-1, -2	HLA Class I	249
Ly49A-C, E-G, I-O	M	C-lectin	ITIM / SHP-1, -2	H-2 class I	240
KLRG1	M	C-lectin	ITIM / SHP-1, -2	?	250
TGF-βR	H,M	CytoR	Smad2	TGF-β family	251
IL-10R	?	CytoR	JAK2, Tyk2 / STAT3	IL-10	252

The activating pathway of NK cells also includes a series of different receptors. Among them, the main ones are Natural Cytotoxicity Receptors (NCRs) whose expression is highly specific to NK cells[216]. It is believed that the main control over the activating pathways of NK cells is regulated by these receptors.

Currently there are three different NCRs identified: NKp30[241], NKp44[243] and NKp46[242]. The expression of these receptors is highly specific to NK cells. However,

the ligands of these receptors have not been characterized yet. NKp30 and NKp46 are expressed both in activated and non-activated NK cells whereas NKp44 expression is restricted to activated NK cells.

Activating receptors do not directly signal through their cytoplasmic tail, but instead associate non-covalently with other molecules containing ITAMs, immunoreceptor tyrosine-based activation motifs, that serve as the signal transducing proteins. NKp30 and NKp46 are associated with CD3ζ whereas NKp44 is associated with DAP12.

Role of NK cells in infections

The importance of NK cells in immunity against viruses is well documented in experimental and clinical settings. NK cells play an important role in host resistance to mouse cytomegalovirus [253]. The reason for this susceptibility is believed to be that some viruses (including CMV) downregulate MHC class I molecules on infected cells[254]. An activating NK receptor, NKp46[255], mediates the killing of influenza virus–infected cells[256].

Another activating NK receptor, CD244/2B4[257] signals by associating with a cytoplasmic adapter molecule called the signalling lymphocyte activation molecule-associated protein (SAP)[258]. Patients who have X-linked lymphoproliferative syndrome were found to have a defective SAP[258], and the NK cells from these patients could not be activated through CD244[259,260]. Intriguingly, these patients are highly susceptible to EBV infection. They often develop fulminating and fatal infectious mononucleosis or B lymphomas, indicating that NK cells may play a role in controlling EBV infection. NK cells have also been implicated in immunity against another Herpes virus—human herpes virus 8 (HHV8)—the virus causing Kaposi's sarcoma. HHV8 encodes viral proteins that block expression of MHC class I, but that also downregulate other cell surface proteins such as CD54 (ICAM-1), that potentiate NK cell recognition and activation[261-263]. A recent study has reported that NK cell function in HIV-infected patients inversely correlates with HHV8 viremia and that response to highly active antiretroviral therapy resulted in enhanced NK cell activity and control of HHV8[264]. Collectively, these studies indicate that NK cells may actively participate in immunity against viruses that cause cancer, such as EBV and

HHV8, and that the NK cell immune strategies and receptors that have evolved to primarily deal with virus infections are applicable to the control of transformed cells.

NK cells in cancer

From the 1980's, when lymphocyte-activated killers were firstly used in end-stage patients with solid tumours, until recent days, many advances have been made in the NK cell field[265]. The idea of using NK cells in therapy is promising, especially in the fields of cancer, SCT and infectious diseases[266,267]. The application of activated NK cells in haematological patients is currently being explored. One strategy is the enhancement of NK cells' anti-neoplastic activity and the infusion of selected NK cells as an alternative to CTLs for GvL and thus avoiding GvHD [265].

NK cells are known to take part in the graft-versus-tumor effect following allogeneic transplantation[268]. This is due to the triggering of NK cell cytotoxicity against the malignant cells as a result of the failure in inhibitory receptor recognition of donor cells due to mismatched HLA haplotypes. In contrast to normal tissue, many tumors express ligands for activating NK cell receptors. This makes NK cells attractive in settings of DLI since they on one hand may mediate GvT reactions while simultaneously will not cause GvHD[269]. Studies have shown that while manifesting a GvL effect, NK cells don't show GvH effect and moreover, T cell-mediated GvHD seems to be prevented by alloreactive NK cells because they eliminate host dendritic cells which initiate T cell-mediated GvHD[270-272]. Thus, allogeneic transplantation of NK cells is a currently used method in treatment of hematological malignancies[273,274].

NK cells also facilitate engraftment and GvL and may ameliorate GvHD by targeting hematopoietic cells of patient origin[271,275]. Thus, since NK cells promote engraftment, exert GvL, and can protect from T cell-induced GvHD, they seem attractive effectors in settings of DLI following SCT against human cancers.

One interesting example of cellular therapy is the NK cell-mediated killing of leukaemia cells[276]. This is based on NK cell alloreactivity and it is crucial to improve engraftment, to prevent GvHD and reduce toxicity. The prevention of GvHD, along with the absence of leukemic relapses, is achieved when HLA-C mismatches exist

between the donor and the recipient. HLA-C mismatches may result in donor NK cells not expressing specific inhibitory receptors to the recipient's HLA-C alleles. Donor NK cells that express for example p.58.1 KIR specific for HLA-Cw2 and HLA-Cw4 alleles, do not match with a recipient expressing HLA-Cw1 and HLA-Cw8 alleles. This way these donor NK cells contribute to the killing of recipient leukemic cells, minimizing the chances of leukemic relapse.

The feasibility of using different autologous effector cell populations that employ different recognition and killing mechanisms has also been investigated. However, the role of NK cells in most of these studies is not fully elucidated. IL-2 with or without LAK cells was initially used as a consolidative immunotherapy after ASCT and at time of minimal residual disease[277,278]. Although, the feasibility of generating IL-2 induced LAK cells early after autologous bone marrow transplantation for AML with promising results was reported[279], this approach was hampered by the IL-2 related side-effects and severe thrombocytopenias[280]. Infusion of NK cells together with the transplant followed by IL-2 administrations[281,282] or post-transplant infusion of NK or LAK cells together with IL-2 have shown the feasibility of these approaches[282-284] however the efficacy is yet to be determined.

NK cells in MM

Little is known about the role and effect of NK cells in MM. Previous reports show increased NK cell counts in the peripheral blood and/or BM of newly diagnosed MM patients[285,286]. A significant increase of CD57 expression, an activation marker, in BM NK cells was observed especially in patients at early stages (I+II)[285] and they were shown to be cytotoxic against the K562 cell line in an extent comparative to peripheral blood lymphocytes whereas samples from healthy donors lacked this cytotoxic response[287]. These results reveal the existence of an increase in NK cell numbers and activation status during early myeloma development which may reflect a host's immune response in an attempt to modulate tumour cell growth. However, neither bone marrow cells nor blood lymphocytes were cytotoxic to autologous and allogeneic fresh myeloma cells even after activation[287] and the activity as well as

number of NK cells were found to decrease throughout the development of the disease[288,289]. Contrary to these findings, myeloma cell lines are susceptible to activated NK cell lysis[290] and there is evidence to suggest that the cytokine activation of NK cells may lead to a better activity against primary myeloma cells[291]. The latter study also suggests that NK cell recognition of primary myeloma cells is negatively regulated by MHC class I molecules, since NK cell susceptibility is enhanced after blocking recognition of MHC class I. Interestingly, the myeloma cell line (derived from pleural effusion) that was used in this study had reduced expression of NKG2D ligands such as MICA, MICB, and ULBP molecules which suggests that progression might be due to the escape of MM cells from NK cell surveillance by down-regulation of NKG2D ligands.

Also, in contrast to these results, it has been recently demonstrated that NK cells from myeloma patients have decreased expression of some activatory receptors compared to healthy controls[292]. Also the same study shows that there's no marked difference in NKG2D expression of NK cells derived from myeloma patients compared to healthy controls[292]. Thus, a more comprehensive study analysing detailed phenotypes of NK cells from myeloma patients compared to healthy controls and their role in autologous anti-myeloma activity is needed. Nevertheless, the clinical response during attempts to use low dose recombinant IL-2 for myeloma treatment[293] suggests that activated NK cells seem to have an inherent anti-MM cytotoxic activity and drugs, such as thalidomide, may further augment this effect[294,295].

Also of importance, are the reports of natural NK cytotoxicity to circulating[296] and ex vivo generated, autologous Ag-pulsed DCs in patients with MM[297]. Immature DCs are efficiently targeted by NK cells while mature DCs are protected by the up-regulation HLA class I. DCs are able to activate NK cells via IL-12 and possibly IL-18, while DC maturation is stimulated by IFN-α released by the NK cell. These observations provide evidence that there is an active interface between the innate and adaptive arms of the immune response that could be harnessed in future anti-MM NK cell and DC based therapies.

Gene therapy

Overview

Developments in molecular biology have led to significant diagnostic methods and the characterization of disorders at the DNA level. In the last three decades, many molecular genetic techniques have been used to get more information about the DNA. The further characterization of the DNA helix, the discovery of restriction endonucleases, the development of vector systems, the cloning of genes, and finally the discovery of RNA interference have been the milestones for gene therapy. Potential targets for gene therapy are hereditary diseases, especially single gene defects as well as acquired diseases such as cancer or certain infections.

Gene therapy is defined as the transfer of genetic material into the cells of an organism to treat disease. Molecular genetic techniques have been used to transfer genetic material into target cells for a diagnostic, preventive or therapeutic purpose. The material transferred may be one or a few genes in size. Gene therapy can be used for germ-line or somatic cells in humans, but for ethical reasons, only somatic gene therapy is currently being performed. The first genetic therapy to humans was attempted during the 1970s[298]. Shope rabbit papilloma virus which produces arginase was infected into children suffering from arginase deficiency. The clinical trial was based on animal experiments showing that the direct injection of a wild type virus could deliver the gene encoding arginase and decrease plasma arginine levels. Unfortunately, the results were not successful. During the 1980s, BM cells of two children with thalassemia were transfected with the herpes simplex virus thymidine kinase (HSVtk) gene as the dominant selective marker by a calcium phosphate precipitation method[299]. The persistence of the transfected gene was observed for up to nine months, but then it became undetectable, probably because of viral vectors used for transferring a foreign gene to the target cells. In 1989, a retroviral vector carrying a marker gene, the neomycin phosphotransferase II (neoR), was used to transduce autologous lymphocytes, and the cells were then infused into the patients in association with immunotherapy for malignant melanoma[300]. In 1990, the first gene therapy experiment with the possibility of producing clinical benefits was initiated[301-303]. Since then, great progress has been made on gene transfer strategies, but new

developments are still required for substantial improvements in current gene transfer technologies.

Gene transfer to HSC

The first gene therapy trials to use retroviral vectors in order to treat a disease, included patients with SCID resulting from adenosine deaminase (ADA) enzyme deficiency, which results in toxic build-up of purine metabolites, apoptosis of early lymphoid progenitors and a SCID phenotype. Culver and colleagues gave patients monthly infusions of mature lymphocytes, transduced *ex vivo* with an ADA-containing MLV retroviral vector. Tests of cellular immunity indicated some improvement coincident with increases in serum ADA. However, concurrent exogenous enzyme supplementation limited the correlation of genetically-modified T cells with possible clinical benefit[303]. The first HSC gene therapy trial demonstrating unequivocal clinical benefit was for the therapy of X-SCID and began in the late 1990s at Necker Hospital in France[304]. X-SCID results from defects in the γ-chain subunit of the IL-2 receptor, also common to other cytokine receptors. In this trial, each patient received autologous $CD34^+$ BM cells, transduced with a standard MLV vector expressing a corrective γ-chain subunit gene from the MLV LTR promoter. The transduced cells were reinfused into these children without prior chemo- or radiotherapy. Rapid and extensive functional T cell and B cell immune reconstitution was observed in all but one child. This success appeared to have overcome almost 15 years of hype followed by disappointment and led the way for much broader applications of HSC gene therapy.

The elation following these positive results was temporary, however, because three of the X-SCID patients eventually developed vector-positive clonal T-cell leukaemias, each approximately three years after the gene therapy. The first two cases developed in the youngest patients in the trial who had received higher cell doses than the rest of the cohort. Molecular analyses of the leukemic cells revealed clonal vector integration near the same genetic locus in both patients: the 5′ end of the LMO2 transcription factor gene. Vector integrations at these sites ectopically increased LMO2 expression[305,306]. When activated by spontaneous translocations, this transcription factor has been linked to human T-cell leukemias[307-309].

Investigators and regulators considered the possibility that the large cell-dosages received by these two patients, combined with specific characteristics of the immature immune system, might have predisposed them to the induction of leukaemia in this specific clinical situation. In addition, the γ-chain-encoding transgene used in this trial might be intrinsically oncogenic, given that signalling through cytokine receptors containing the γ-chain can enhance leukemogenesis[310]. Viral integration into the LMO2 locus has also been shown to cooperate specifically with insertions activating the γ-chain in mouse leukaemias resulting from productive infection with replication-competent MLV and suggesting a particularly high risk of LMO2 insertion for vectors expressing the γ-chain transgene[311]. Extrapolation of these results to older patients with X-SCID or patients receiving retrovirally-transduced $CD34^+$ cells for other disorders was difficult. Investigators were reassured by the fact that no patient in the ADA-SCID trial developed leukaemia and eventually, clinical trials using MLV vectors for disorders other than X-SCID were allowed to proceed. However, a third patient from the X-SCID trial has recently developed leukaemia, despite being older and receiving a lower $CD34^+$ cell dose.

Gene therapy researchers have recognized that inserting the proviral form of retroviral vectors into the genome could lead, via their own regulatory elements, to a variety of dysregulated gene expression patterns including gene activation by viral enhancer or promoter activity, post-transcriptional dysregulation through altered splicing or polyadenylation or insertional inactivation of tumour suppressor genes[312]. Administration of replication-competent MLV to susceptible mouse strains has led to tumour development and outgrowth of vector-positive clones[313-315]. Although the tumorigenic genes in this model were often oncogenes, genes involved at almost every stage of growth regulation were also identified; tumours were caused by gene activation more frequently than by inactivation.

The risk of insertion by replication-defective vectors into genomic sites capable of contributing to transformation was initially estimated to be extremely low[316], based on the assumption that proviral integration into the genome was random and that most cells had only one insertion per cell. Modelling based on the size of our genome, the 10 kb gene activation window for retroviral integration and the estimate that there are

~1000 proto-oncogenes gives a probability of an insertional event within 10 kb of a potential proto-oncogene as 0.001–0.01[317]. However, clinical and experimental data suggest that only a few genes might be open to the retroviral integration machinery at each stage in a cell's development, potentially owing to the chromatin environment around differentially expressed genes[317].

Many researchers believe that vectors based upon lentiviruses, the class that includes human, simian, feline, and bovine immunodeficiency viruses, might have advantages in terms of gene therapy efficiency and control over gene expression[318]. However, it remains unclear whether using lentiviral vectors will reduce the risk of insertional mutagenesis. Patients naturally infected with HIV have repeated insertion events in their T cells without reported cases of T-cell leukaemias containing HIV. However, natural HIV does not infect HSCs or other progenitor cells that might be more susceptible to transforming insertions. In the NOD/SCID model, lentiviral vectors transduced human SCID repopulating cells with high efficiency and multiple copies of the transgene were detected per cell, potentially increasing the risk of insertional mutagenesis[318].

The sleeping beauty transposon (SBT) is another potential non-viral method for genetic addition[319]. Non-viral gene therapy approaches, such as SBT, avoid several of the problems that researchers working with many retroviruses encounter, including viral immunogenicity and the requirement to engineer permanent replication-incompetent stocks. Because functional SBT is simply a molecule of naked DNA encoding the transposase enzyme and a therapeutic gene, which can be very large, SBT could avoid the intractable problem of immunogenicity. However, because of the random nature of SBT integration, it is still unclear whether its use will decrease the risk of insertional mutagenesis. Finally, non-integrating vectors that replicate as episomes are an attractive approach, allowing passage of the vector into daughter cells without the risks associated with chromosomal integration[320]. The problem with these non-viral vectors is cellular entry: electroporation and transfection are toxic, and inefficient for transfer to primary haematopoietic cells, whereas micro-injection of individual cells is impractical.

One other aspect to consider is the number of integration events transplanted into patients. It is probable that multiple insertions into single cells will result in a disproportionate increase in the risk of insertional mutagenesis[311]. Most models of tumorigenesis indicate that multiple hits are required for full cellular transformation; therefore, it is reasonable to aim for one integrant per cell. However, with the development of gene transfer vectors with a higher efficiency of integration and higher titres [allowing superior multiplicity of infection (MOI)], cells with more than one integrant are unavoidable. This could fuel dangerous transformations by contributing additional hits, potentially activating unchecked growth according to the multistep model of tumour initiation and progression[321].

Therefore, new strategies designed to minimize the consequences of such events are needed. Construction of bicistronic vectors carrying both therapeutic and suicide genes would enable selective elimination of uncontrolled proliferation of transduced lymphocytes[322]. This way, any undesired activity such as malignant transformations or GvHD caused by gene modified cells might be controlled.

Gene transfer into lymphocytes

The safety of retroviral gene transfer into lymphocytes has also been evaluated both in animal models and in clinical protocols of somatic gene therapy. No local or systemic toxicity related to the gene transfer procedure has been observed so far.

The representation of different gene ontology categories in the hit genes in T cells before and after DLI seem to be similar to the expected values for a random distribution. Interestingly, a slight overrepresentation of proliferation-related genes in uninfused T cells disappeared in T cells obtained from patients, suggesting that such hits have, if anything, a negative effect on in vivo survival of transduced T cells. In cultured T cells, integrations into active genes, in direct transcriptional orientation, or upstream from transcription start sites, are significantly underrepresented, suggesting that, in cells subjected to strong selective pressure, interference with transcription, splicing, and polyadenylation is more likely to lead to clonal ablation than expansion. Preliminary results indicate that transduced T cells are highly polyclonal, with vector integrations occurring preferentially within genes.

A crucial parameter in the calculation of the potential risks of gene transfer is the frequency by which an integrated provirus leads to activation, or deregulation, of gene expression. When the expression of targeted genes in randomly selected T cell clones were analysed, it was found that almost 1/5 of them are up-regulated independently from the distance of the integrated vector, which varied from 25 kb upstream to >100 kb downstream of transcription start sites, and its transcriptional orientation. Most up-regulated genes are expressed at very low levels or not expressed in T cells, suggesting that proviral insertion is more likely to affect genes that are not already engaged in high-level transcription. These findings suggest that insertional gene activation is not simply a function of relative distance or orientation of integrated proviruses but may depend on the permissiveness of gene regulatory elements to the activity of transcription factors recruited by the LTRs.

Overall, numerous analysis in different clinical trials indicate that genetic modification of T cells with retroviral vectors is safe and is not associated to a measurable risk of insertional oncogenesis. This finding is in contrast with that observed in the X-linked severe combined immunodeficiency trial and reinforces the concept that the risk of gene therapy in general, and of the use of retroviral vectors in particular, must be assessed in each specific experimental and clinical context. This analysis should include the biology of the target cell, the cell activation and transduction procedure, and the nature and function of the transgene, as well as the vector design. Given the therapeutic potential of gene transfer technology, there is no alternative to its testing in carefully designed clinical trials, where the risk-benefit balance must remain the central consideration.

Suicide gene therapy

The theory behind this approach is based on the expression of a “suicide gene”, often coding for an enzyme that converts a non-toxic prodrug to a toxic product. This results in the selective killing of these cells upon administration of the prodrug. The key point in this approach is that the prodrug is not processed in normal cells, whereas it is converted to a toxic end product by those cells that have the suicide gene for catalysing this conversion.

There are several different suicide genes being used for cancer therapy or the control of GvHD during transplantion[323-329] but the best defined and most widely used suicide gene is HSVtk[330]. HSVtk converts the non-toxic guanosine analogue Ganciclovir (GCV)[331] to GCV-monophosphate and this molecule is further processed by endogenous kinases to produce GCV-triphosphate. This final product competes with normal GTP nucleotides and acts as a chain terminator during DNA replication, causing cell cycle arrest and death via apoptosis in dividing cells[332].

Our group has previously demonstrated that this toxic product is transferred to neighbouring cells via gap junctions and induces apoptosis there (a phenomenon called the "bystander effect")[333]. This phenomenon has also been confirmed by other studies[334-337]. Moreover, *in vivo* studies have taken this one step further by showing that even in the absence of gap junctions, such as two anatomically separated tumours with no known metabolic cooperation, the suicide gene therapy applied to one of the tumours also has effect on the second one. This is the so called "distant bystander effect"[338-340] which is the result of an immune response [341,342].

The HSVtk/GCV approach has been used in several clinical trials. For example, HSVtk transduced donor T cells were infused to relapsed leukemic patient after allogeneic SCT to obtain maximum GvL effect and GCV administration. This method allows eradication of the gene modified T cells in case of GvHD[343-345]. In this trial, some HSVtk transduced T cells have survived for 10 years after gene therapy and are still sensitive to GCV[346].

Escape mechanisms of suicide gene such as chromosomal deletion and silencing of HSVtk gene can influence the outcome of GCV treatment[347]. Silencing of HSVtk gene is a serious problem because it results in continuous cell proliferation even after GCV treatment. However, gene silencing can be overcome, using for example the MSCV LTR-based vectors engineered to resist de novo methylation[348-350].

Gene marking

The principle of gene marking is the transfer and integration of a unique DNA sequence (e.g. a non-human gene) into the DNA of a host cell (e.g. T cell, or

haematopoietic stem cell) allowing the gene or gene product to be easily detected, thereby serving as a marker for these labelled cells[351].

Early gene transfer trials involving humans were mostly gene marking studies, with the objectives not with any direct therapeutic intent, but to test feasibility and safety of oncoretroviral gene transfer to repopulating hematopoietic cells in patients undergoing high dose cancer chemotherapy with autologous stem cell rescue. The neoR gene, which can be detected by PCR or by determining the frequency of hematopoietic colonies resistant to the neomycin analog, was used as a marker gene in most trials. The results of the first gene marking study were published in 1990[300], and it was followed by a wide variety of investigations that marked HSCs or their progeny[352-354]. For example, ASCT had shown promise as an effective treatment for leukemias and lymphomas, and some solid tumors, but disease recurrence was the major cause of treatment failure[355,356]. When the tumor originated from or involved the BM, relapse could originate from malignant cells persisting in the patient, in the rescuing HSC, or in both. Concern that the HSC may contain residual malignant cells led to extensive evaluation of techniques for purging these cells. However, it was hard to show that this reduced the risk of relapse, and the purging techniques usually slowed engraftment and reduced long-term hematopoietic reconstitution after transplant. Gene marking was subsequently used in a series of protocols that demonstrated the safety, persistence and effectiveness of viral- and tumor-specific T lymphocytes, and provided invaluable guidance as to necessary dosage, persistence and efficacy.

Gene marking strategies were successfully used to answer the question whether autologous BM cells, harvested from patients in remission from malignant disease and reinfused as stem cell rescue following high-dose chemotherapy, could contribute to relapse of disease, or whether the relapse originated exclusively from residual malignant cells in the patients. Gene marking allowed investigators to study sites of adverse events, and determine whether infiltration of the gene-marked cells had contributed to the pathology when studies clearly showed that the harvests were contaminated by malignant cells capable of contributing to a relapse, as a proportion of the recurrent malignant cells carried the neoR gene[351,354,357]. None of these data could have been obtained so definitively by conventional means, and they have

allowed immunotherapy to play an increasing role in the treatment of viral infections and cancer.

But even given the safety and value of gene marking over the past two decades, we can't really be certain that it is not capable of producing the same severe adverse events as those observed in the French trial. The only thing certain about any and every medical practice is that it is never completely safe. However, the risks of gene marking, based on theory and observations, should remain very low. Gene marking occurs at a substantially lower level than gene correction in long-term follow-up patients, marking levels in T cells and hematopoieitic cells are extremely low. The chance of multiple oncogenic integrants under these circumstances is remote. Unlike correction of γ chain SCID, gene marking does not lead to selection *in vivo* for transduced cells, nor does it select for a subset of cells in which the transgene is produced at high level. Perhaps most importantly of all, unlike correction of SCID, marking leads to no hyper-expansion of corrected T cells. In patients who received gene-marked BM or T cells and were followed for up to a decade, none have shown any evidence for selective expansion of marked cells. Instead, in all patients, the percentage of marked cells gradually drifts downwards.

Aims of this study

The main goal of this thesis was to identify strategies for effective immunotherapy in MM.

The specific aims leading step-by-step to our main conclusion are listed below.

I) To analyse the long term effects of retroviral gene transfer and address whether the myeloma cells re-introduced to the patient during ASCT might have contributed to the relapse.

II) To establish an experimental immunocompetent multiple myeloma model where gene marked MM cells can be traced and quantified *in vivo* for studying the effect of different treatment modalities.

III) To identify the effector cells that are capable of autologous tumor cell killing after IL-2 administration in C57BL/KaLwRij mice and to use these cells for MM therapy through adoptive transfer.

IV) To generate a system for specific autoactivation and stimulation of NK cells through the expression of genetically engineered IL-2 variants.

Methodology

Patients

The patients in the clinical study (paper I) were considered individually based on careful review of past medical history and present status. The protocol was offered to those patients that had been diagnosed with multiple myeloma which were intended to undergo high-dose chemotherapy and ASCT. The patient's decision to participate in the study or not, did not alter the type or time point of ASCT. The study was approved by the ethics committee at Karolinska University Hospital, Huddinge in Stockholm, Sweden and the Swedish medicinal products agency.

Cells

Cell lines and cell culture conditions

The **5T33** murine myeloma cell line used in paper II and paper III was derived from C57BL/KaLwRij and kindly provided by Dr J. Radl (TNO Institute, Rijswijk, The Netherlands). The growth medium used for these cells was minimal essential medium containing 2mM L-glutamine, 100mM non-essential amino acids, 100 mM sodium pyruvate, 5 x 10^{-5} M 2-mercaptoethanol and 10% heat-inactivated fetal calf serum (FCS). The cells were maintained at 0.5x10^{6} cells/ml concentration monitored daily. 5T33 MM cells transduced with retroviral vector containing HSVtk gene were tested for GCV sensitivity.

The **YAC-1** cell line used in paper III was grown in RPMI 1640 medium with 10% FCS (all components were purchased from GIBCO-Invitrogen, Invitrogen AB, Stockholm, Sweden). YAC-1 is a mouse lymphoma cell line which is sensitive to the action of NK cells and are useful in assays of NK cell activity.

The **Phoenix GP** retrovirus packaging cell line (with permission from Dr Garry P. Nolan, Ph.D., Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA, USA) used in papers II, III and IV and **Cos-7** cell line

(DSMZ, Braunschweig, Germany) used In paper IV were cultured in DMEM with 4500 mg/ml glucose, pyridoxine, Na-pyruvate and Glutamax-II with 10% FCS.

The **NK-92** cell line used in paper IV was purchased from LGC Promochem/ATCC (Borås, Sweden) and maintained in stem cell medium (CellGro; CellGenix, Freiburg, Germany) supplemented with 10% FCS and 500 IU/mL Proleukin (Chiron, Emeryville, CA, USA).

The **K562** human chronic myelogenous leukemia cells (LGC Promochem/ATCC) were used as a target for NK cells in paper IV and cultured in RPMI 1640 medium (Invitrogen), supplemented with 10% FCS.

All cell lines were incubated at 37°C, 5% CO_2 and 95% humidity and medium was changed every 2 to 4 days. Aliquots of early passaged cells were frozen in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), 90% FCS and stored at -180°C for later reconstitution. All cell populations were observed at regular intervals using an inverted microscope (Olympus CK40) equipped with a UV module at regular intervals and were monitored regularly for viability with Trypan blue dye exclusion and for mycoplasma contamination.

Primary cells

In paper I, at the time of BM and blood cell collection, half of the cell harvest was left untreated, frozen and stored. $CD34^+$ cells were sorted from the rest of the harvested blood and marrow samples and subjected to retroviral gene transfer.

Mouse NK cells (paper III) were cultured in the complete RPMI-1640 medium [RPMI-1640 medium supplemented with 10% inactivated FCS, 2 mmol/L L-glutamine, 25 mmol/L $NaHCO_3$, 1 mmol/L sodium pyruvate, 25 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 100 kU/L penicillin G, and 100 mg/L streptomycin] with Trace Elements A (Mediatech Inc., Herndon, VA). Proleukin® (rIL-2) in fresh complete RPMI medium was added on day 0 and every other day afterwards. For expansion, they were cultured in a concentration of 1×10^6 cells/ml and the cell density was determined daily.

Cell separation

CD34-sorting in paper I was done by cell centrifugation and concentration of the buffy-coat fraction of the cells followed by two times washing in phosphate buffered saline (PBS) and incubation with anti-CD34 IgM antibodies. Antibody-bound cells were sorted using the CellPro Ceprate SC avidin-biotin column.

Also in paper I, myeloma cells of relapsing patients were enriched by magnetic cell separation before marker gene analysis, due to the relatively low ratio of myeloma cells to normal BM cells at the time of relapse. BM cells were separated by density gradient. The cells were then incubated with the mouse-monoclonal antibody BB4 (Serotec), after which the BB4 positive cells were separated with immunomagnetic beads. With this method, a nearly pure population of myeloma cells was obtained from the BM of the patients that was analyzed by PCR for the presence of the transgene in tumor cells in relapse.

For the selection of eGFP-transduced 5T33MM cells in paper II, the GFP bright cells were sorted on a FACS Diva (Becton-Dickinson, Franklin Lakes, NJ, USA) at three sequential occasions. MO3TIN (HSVtk)-transduced 5T33MM cells were selected in the presence of 1mg/mL G418 (Invitrogen, Paisley, UK) for 1 week.

In paper III, after preparation of single-cell suspensions, mouse NK cells were separated using a CD49b (DX5) Mouse Microbeads Kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. In this study, the $DX5^+$ separated-NK cells were used for cytotoxicity assay, retroviral transduction and adoptive transfer.

Flow cytometry

The phenotype of the cell lines, peripheral blood mononuclear cells(PBMC) and single cell suspensions from different mouse organs (spleen, liver, BM, and thymus) were analyzed using a FACSCalibur flow cytometer (papers I, II, III, IV). Four-color fluorescence was used according to standard procedures. Cells were mixed with

appropriate concentrations of different dye-conjugated monoclonal antibodies, incubated for at least 15 minutes at room temperature and finally washed with PBS prior to flow analysis. Human NK cells were defined as $CD56^{+}CD3^{-}$, T cells as $CD56^{-}CD3^{+}$, and both NK and NKT as $CD56^{+}$. Mouse NK cells were defined as $NK1.1^{+}$ and/or $DX5^{+}$. Propidium iodide (PI) staining was used for dead cell exclusion. In each sample, at least 10.000 cells were acquired in the analysis region of viable cells, defined by side and forward scatter.

ELISA

For the quantitative determination of human IL-2 (paper IV), mouse IL-6 (paper II) and mouse IL-2 (paper III), the OptEIA Human IL-2 ELISA Kit II, the murine IL-6 and IL-2 BD OptEIA ELISA Kit, respectively, were used according to the manufacturer's (BD Biosciences, CA, USA) instructions. The interleukin levels were determined by comparing the optical density results to standard curve using recombinant IL-2 or IL-6 provided by the manufacturer.

In paper II, the levels of IgG2b in the cell culture supernatants were measured by ELISA. Micro-ELISA plates were coated overnight at $4^{o}C$ with goat anti-mouse IgGb in carbonate buffer pH 9.8. The plates were then blocked for 2 hours at room temperature with PBS containing 1% bovine serum albumin. After blocking, the samples or standard IgG2b in serial dilutions with PBS containing 1% Tween-20 were added, and the plates were incubated overnight at $4^{o}C$. Thereafter, the plates were washed three times with PBS containing 1% Tween-20. For IgG2b measurement, the plates were incubated with goat anti-mouse IgG2b for 2 hours at RT. Finally, all the plates were developed. After 20 to 30 minutes of incubation at RT, the color reaction was quantified by an ELISA reader at 405 nm.

Retroviral vectors

Construction of vectors

The replication deficient G1Na vector used in paper I was constructed at Genetic Therapy, Inc. The bacterial neoR gene was inserted into a modified Moloney murine leukemia virus (MoMLV) backbone. The vector had a number of modifications to minimize the risk of recombination between the vector and packaging construct, that would result in creating replication-competent viruses.

The retroviral vector plasmid pSF91-DsRed2-N1-g used in paper III was created by cloning the DsRed2 gene from pDsRed2-N1 (BD Clontech, CA, USA) as an EcoRI-MfeI fragment into the EcoRI site of pSF91-MCSg[358].

In paper IV the pORF-hIL2 plasmid, containing the IL-2 cDNA template, was purchased from InvivoGen (San Diego, CA, USA). The required IL-2 primers were designed using Oligo 6.6 software (Molecular Biology Insights Inc, Cascade, CO, USA). They were ordered from DNA Technology, Aarhus, Denmark. The IL-2 variants were amplified by PCR. The PCR products were cloned into pCR4BluntTOPO vector (Invitrogen). The TOPO cloning and transformation steps were performed according to manufacturer's instructions. Clones were characterized by using restriction analysis and cycle sequencing and subcloned with EcoRI into pSF91-MCSg. pSF91-MCSg was derived from the mouse leukemia virus–based retroviral vector pSF91-GFP-gPRE, a kind gift from Prof. C. Baum (Hannover Medical School, Hannover, Germany). Finally, three constructs were prepared: one expressing wild-type IL-2 (secreted), one targeted to cytoplasm, and one targeted to the ER.

For eGFP vector construction which is also used in papers II, III and IV, the eGFP gene from pEGFP-N3 (Clontech, Palo Alto, CA, USA) was released with HindIII-NotI (filled in) and inserted into pSF91-MCSg between HindIII-SalI (filled in), and the resulting plasmid was called pSF91-GN3g. All constructs were confirmed by restriction mapping and partial sequencing.

The plasmid pMO3TIN used in paper II, containing the HSVtk and NeoR genes linked by poliovirus IRES in the MoMLV backbone, was kindly provided by Dr Klaus Kühlke (EUFETS, Idar-Oberstein, Germany).

Retroviral vector production and transduction

The G1Na-producer cell line for paper I was created by transfection of the vector into the PA317 producer cell line. The vector containing supernatant obtained from the producer cell line was extensively tested for exogenous pathogens and replication-competent virus. The supernatant (provided by Genetic Therapy, Inc.) to be used for human clinical studies had passed all quality controls. The cells were exposed to the G1Na vector at a ratio of 5 vector particles per cell. The cells were incubated in vector-containing supernatant at 37 °C in the presence of stem cell factor (100 ng/ml), interleukin-3 (20 ng/ml), interleukin-6 (100 ng/ml), basic fibroblast growth factor (20 ng/ml) and protamine sulphate (4 µg/ml). After 12 hours incubation, the cells were washed twice in 1xPBS. The incubation in fresh vector-containing supernatant was repeated twice, with 6 hour incubation periods.

For the production of retrovirus in papers II, III and IV Phoenix GP cells were transiently transfected with 3µg of vector construct plasmid (IL-2WT, IL-2ER, GFP and DsRed) and 1µg pMD-G (encoding vesicular stomatitis virus envelope glycoprotein) per 35 mm cell culture well, using the Fugene 6 reagent according to manufacturer's instructions in papers II-IV. Virus supernatants were collected from transfected PhoenixGP cells 24 and 48 hours after transfection, filtered through 0.45µm Millex-GP syringe-top filter and used immediately for transduction of PG13 retroviral packaging cell line. The obtained virus titers from PG13 varied up to 10-fold (10^5-10^6 virus particles/ml). These vector-containing supernatants were used to transduce different cell types, such as NK-92 (paper IV), 5T33MM (paper II and III) and $DX5^+$ NK mouse cells (paper III), by centrifugation at 1000xg for one hour in the presence of 4µg/ml polybrene.

In all cases viral titers were estimated indirectly by transducing HeLa cells with different volumes of retroviral-containing supernatant. Increasing volumes of

supernatant (2, 20 and 200 ml) were plotted against the percentages of fluorescent target cells determined after 48 hours by FACS analysis. Titers were calculated according to the following formula: Titer (HeLa-transducing units/ml) = (number of HeLa cells infected) x (% of positive cells/100)/volume of supernatant (in ml).

Polymerase chain reaction

In paper I genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). PCR amplification was performed in 50 µl volume containing 10 pmol of each primer, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.3mM dNTPs buffer, and 1.25unit Taq polymerase from Gene Amp kit from Perkin-Elmer (Branchburg, New Jersey, USA). One µg of genomic DNA from BM or peripheral blood sample was used. At every time point of receiving peripheral blood samples from the patients, peripheral blood samples extracted concurrently at the hospital blood bank, was used to prepare a negative DNA control for the PCR analysis. Genomic DNA from BB4 (CD138) separated myeloma cells obtained from relapsed patients were also analyzed with the same protocol.

One tenth of the PCR product was subjected to nested PCR. Five µl were transferred to a new 50µl the same reaction buffer including this time internal primers.

For quantitative PCR, 10 µl of DNA were subjected to a duplex TaqMan PCR with primers and probes specific for neoR and human Epo Receptor. In the PCR run, DNA from mixtures of HT1080 cells containing a single copy of a retroviral vector carrying the neoR gene (HT1080Mo3TIN) with wild type HT1080 cells were used as positive controls.

Studies with in vivo models

Mice

Female and male C57BL/KaLwRij mice were purchased from Harlan CPB (Horst, The Netherlands). C57BL/6 mice were from the Microbiology and Tumor Biology Center breeding unit at Karolinska Institutet. Both strains were housed in our animal

facilities at the Clinical Research Centre at Karolinska University Hospital, Huddinge, Sweden under conventional conditions including access to tap water and standard chow *ad libitum*. All mice were 8 to10-weeks old at the beginning of each experiment. The study was approved by the local animal ethics committee in south Stockholm, Sweden.

Tumor cell injection, induction of MM and IL-2 administration

In papers II and III, groups of C57BL/KaLwRij mice were injected intravenously (i.v) with 10^5 eGFP- 5T33 MM (for biodistribution studies) and/or non-transduced 5T33 MM cells suspended in a total volume of 100 µl sterile PBS/mouse. Control mice were injected with an equal volume of PBS i.v. The animals were examined twice daily for the development of paraplegia.

In paper III, rIL-2 (Proleukin, Chiron) was injected intraperitoneally (i.p.) at a concentration of 600 µg/kg (~$9.83x10^6$ IU/kg) and administered twice daily for a total of 14 days. The mice were then monitored for disease development.

In vivo depletion of mouse $NK1.1^+$ and $CD8^+$ cells

In paper III, to deplete $NK1.1^+$ cells *in vivo*, mice were injected starting 2 days before MM challenge with 200 µg i.p. of anti-NK1.1 monoclonal antibodies (mAb), and every 5 days thereafter, until termination of the experiment, with 200 µg i.p. of the anti-NK1.1 mAb. Control mice were injected with a similar volume (0.2 ml) and dose of mouse IgG antibody (Sigma) as an isotype control. In parallel, cytotoxic T lymphocytes were depleted *in vivo* by using purified anti-CD8 MAbs (Clone 2.43); 0.5 mg per mouse of antibody was injected i.p. every 5 days from day −2, until the endpoint. The efficacy of depletion of $NK1.1^+$ or $CD8^+$ cells was monitored by flow cytometric analysis of spleen cells at the endpoint. Animals exhibiting $NK1.1^+$ or $CD8^+$cells in the spleen were excluded from the study ($n = 1$ for NK1.1 and n=3 for CD8 depletion).

Post-mortem analysis of mice

At weekly intervals and at the time of myeloma development, the mice were killed by CO_2 inhalation followed by cervical dislocation. For biodistribution studies the spleens, livers, thymi and lymph nodes were then excised and kept in PBS until processing for preparation of single-cell suspensions. BM from femora and tibiae were obtained by flushing PBS into the cavities of bones.

Single cell suspensions of spleen cells from mice were pooled in serum-free RPMI-1640 medium by filtering the suspension through mesh with the aid of a homogenizer to exert gentle pressure on the spleen fragments. Erythrocytes were lysed in ammonium chloride solution [0.15 mol/L NH_4Cl, 10 mmol/L $KHCO_3$, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.2] or were separated using Lympholyte M (Cedarlane labs, Ontario, Canada).

Adoptive transfer of NK cells

After transduction, transduced NK cells were sorted into $DsRed^+$ (transduced) and $DsRed^-$ (untransduced) populations by a FACS sorter (BD DIVA, BD Biosciences, CA, USA). These two effector populations, as well as NK cells that were only cultured without transduction, were separately injected i.v., at doses of $2x10^5$ or $20x10^6$ cells/mouse, into C57BL/KaLwRij mice that had been inoculated with 10^5 5T33MM or eGFP-5T33MM cells on the previous day. Mice were then monitored for survival, and flow cytometric analysis was performed.

Cytotoxicity assay

The cytotoxic function of NK cells was measured by a standard 4 hour ^{51}Cr-release assay during different timepoints of culturing. As targets, the NK-sensitive K562 cell line (paper IV), and in papers II and III, the 5T33MM, YAC-1 cell lines and fresh autologous cells, were used. Target cells were labeled with 100µl ^{51}Cr and incubated for one hour at 37°C. Effector cells were counted using trypan blue dye exclusion and mixed with target cells to obtain an effector: target ratio of 10:1, 3:1, 1:1 and 0.3:1. Culture medium was used as a negative control, and for a positive control cells were

incubated with 1% Triton X. After incubation in a V-bottom shaped 96-well plate for 4 hours at 37^{o}C, 70µl of each supernatant was aspirated from each well and counted in a gamma counter. The percentage of spontaneous release was calculated from the following formula: % specific ^{51}Cr release = (sample release-spontaneous release) / (maximum release – spontaneous release) x 100.

Results

Lack of complete eradication is probably the cause for MM relapse

In this study, we analysed if the contribution of autologous infused cells to relapse as well as the long term persistence of a transgene in haematopoietic cells following ASCT for MM, we genetically marked autologous $CD34^+$ enriched bone marrow or peripheral blood cell grafts from eight myeloma patients using retroviral vectors (paper I). The average transduction efficacy of $CD34^+$ enriched cells was 2% (0.43% - 5.1%) (Table 3). The median total cell count was $3.53x10^{10}$ before and $2.46x10^8$ after separation. Average transduction efficacy of $CD34^+$ enriched cells was 2% (0.43% - 5.1%). Patient 2 died before transduction and patient 3 died before ASCT.

Table 3: Separation, transduction and transplantation of $CD34^+$ cells.

Patient #	1	2	3	4	5	6	7	8
Initial total cell count ($x10^6$)	5500	42000	43500	33000	47100	37500	14200	21480
Initial $CD34^+$ count ($x10^6$)	7	NS	374	1683	636	994	328	752
$CD34^+$ after separation ($x10^6$)	5	NS	262,9	304	180	213,2	49,1	187,6
Number of $CD34^+$ cells for transduction ($x10^6$)	1,9	NS	51,5	268	39,2	70,8	19,5	77,2
Number of colonies /10^6 cells	13600	8000	34600	10600	77600	30300	32300	183300
Transduction efficacy (% colonies)	2,37	NA	5,1	4,02	0,43	1,91	2,06	0,73
Transduced cells for transplantation	104280	NA	NA	125840	39775	417000	1370000	591300
$CD34^+$ cells transplanted ($x10^6$/kg)	4.87	NA	NA	5.66	2.16	3	1.95	3.37

Following the transplantation of gene marked $CD34^+$ enriched cells, we were able to detect the transgene until the end of follow-up, 5 years after ASCT. The existence of transgene was firstly confirmed with colony assays up to 6 months, after which they were no longer detected by this method. More sensitive nested-PCR method was still able to detect the transgene until the end of follow-up. Moreover, the number of marked cells was determined by Q-PCR and was found to be less than 1 in 10^5 cells at the end of the follow-up. The expression of the gene was also followed by RT-PCR but the sensitivity of the method did not allow the detection of any expression from such small number of positive cells at the end of the follow-up. Furthermore, we

analyzed if the CD34 enriched cell population could include non-detectable contaminating transduced myeloma cells, later giving rise to relapse. We analyzed biopsy material from three relapsed patients using nested PCR and the results showed that the transgene was expressed only in normal cells and not in myeloma cells. The results of transgene follow-up during the 5 year period by the use of above mentioned methods is summarised in Table 4.

Table 4: Colony assays and PCR analysis for neoR sequences of bone marrow cells following autologous transplantation.

Patient #	1	4	5	6	7	8
Time period	**Positive colonies**					
3 weeks	2%	1%	4%	1%	1%	1%
3 months	NA	1%	5%	1%	5%	1%
6 months	2%	2%	2%	0%	1%	0%
PCR	**Normal PCR/Nested PCR positive (N+) or negative (N-)**					
3 weeks	+	- / N+	NA	+	+	+
3 months	+	NA	- / N+	- / N+	+	+
6 months	+	- / N+	- / N+	-/N+	+	-/N-
Nested PCR						
1 year	+	+	+	D	+	-
2 years	+	D	+		+	D
3 years	+		+		+	
4 years	NA				+	
5 years	+		D		NA	

Our results suggest that the myeloma cells in relapse may not have been generated from the infused gene modified cells. Tumor biopsies from relapsing patients were analyzed and no MM cells expressing the marker transgene were detected. The reliability of transgene analysis was confirmed by positive transgene expression detection in normal BM cells of the patients for up to five years post-transplant. No toxicity attributable to marker gene or vector was observed. These findings support that the lack of complete eradication of residual tumor cells by conditioning rather than the re-introduced myeloma cells is the cause of relapse and therefore, new treatment modalities are needed.

Visualisation of 5T33 MM cells in the C57BL/KaLwRij mouse

Based on these findings, we decided to return to experimental models where we can set up a model to analyse novel myeloma treatment modalities. We established a murine model of MM (paper II) that makes it possible to track myeloma cells *in vivo* by using eGFP expressing 5T33 MM cells for induction of MM in C57BL/KaLwRij (H-2^b) mice.

In order to obtain a highly pure population of eGFP expressing MM cells, 5T33 MM cells transduced with eGFP were sequentially sorted three times by FACS. Then, the purity of the transduced cells, as well as the eGFP expression, was determined by flow cytometric analysis. The selected eGFP-transduced cells could reach up to 99.81% purity with high expression of eGFP when compared with parental nontransduced 5T33 MM cells.

The gene modified cells were compared in many respects to the normal nontransduced cells and it was confirmed that there was no detectable differentiation in the behaviour of these cells due to genetic manipulation. The doubling times and/or proliferation rates of 5T33 MM cells transduced with eGFP were very similar to that of nontransduced parental cells, suggesting that transduction of 5T33 MM cells with this retroviral vector containing a foreign gene does not affect the growth rate of these cells.

Supernatants of cell cultures, regardless of whether they were obtained from transduced or nontransduced 5T33 MM cell cultures, contained similar concentrations of IgG2b and IL-6. Cells were highly tumorigenic and could induce the MM associated symptom (paralysis in the hind paws) in C57BL/KaLwRij mice being observed approximately 22 to 43 days (depending on the injected cell numbers) after cell inoculation (Figure 2).

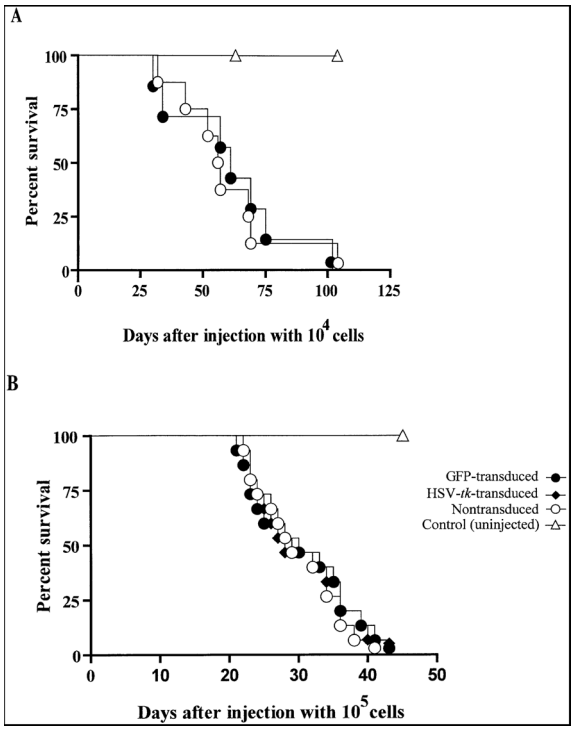


Figure 2: Retrovirally transduced 5T33MM cells induce MM survival kinetics similar to nontransduced cells.

More importantly, no significant differences in survival times were observed when tumor inoculation was performed with transduced or nontransduced 5T33MM cells ($p > 0.3$). The median survival times for animals inoculated with 10^5 transduced cells were 30 (mice injected with eGFP$^+$ cells) and 28 (mice injected with HSVtk cells) as compared with 29 days for animals injected with same numbers of nontransduced cells.

With this model, it was possible to track the marked myeloma cells and analyze their distribution during the course of the disease. High numbers of eGFP$^+$ cells could be readily detected in the BM, spleen, and liver. However, other tested organs either contained very low numbers (lymph nodes) or were devoid of (thymus) eGFP$^+$ transduced cells (Figure 3). The BM contained the highest numbers of eGFP$^+$ cells, which indicates that these cells, like the nontransduced parental cells, predominantly localize in BM.

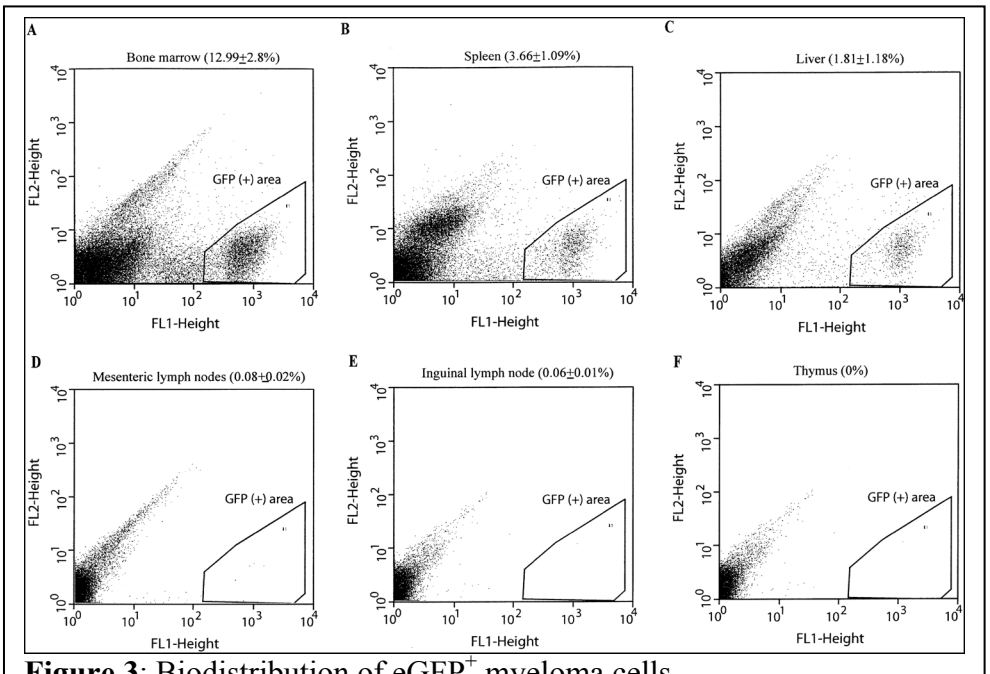


Figure 3: Biodistribution of eGFP$^+$ myeloma cells.

IL-2 administration prolongs survival of 5T33 MM bearing mice in an NK cell dependent manner

One of the therapeutic effects we wanted to analyse was administration of IL-2 in these mice (paper III). Effector cells in peripheral blood against mouse autologous multiple myeloma target cells have, in previous reports, shown limited cytotoxicity that could be increased after IL-2 activation. Therefore, using this mouse model, we aimed to identify the effector cells in peripheral blood that are responsible for autologous myeloma cell killing and to use these cells for adoptive immunotherapy.

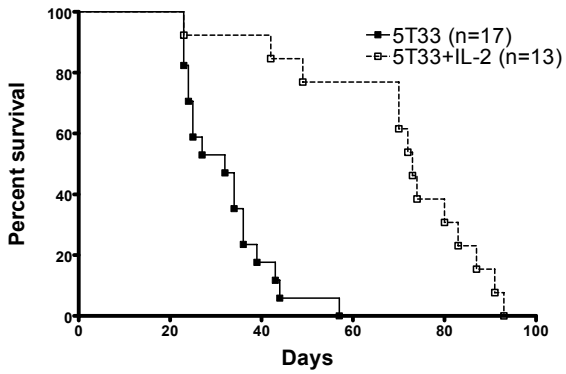


Figure 4: Survival kinetics of 5T33MM inoculated C57BL/KaLwRij mice receiving IL-2 therapy.

To test if IL-2 prolonged the survival of 5T33 MM bearing mice, mice were inoculated with IL-2 twice daily. IL-2 administration into 5T33 MM bearing C57BL/KaLwRij mice significantly prolonged their survival (Figure 4).

This observation led us to test if the prolongation of survival observed after IL-2 treatment was dependent on NK cells, $CD8^+$ T cells, or both. Interestingly, specific depletion of NK cells, but not $CD8^+$ T cells, abolished the IL-2-dependent anti-tumour effects in 5T33-inoculated mice (Figure 5). These results indicate that NK cells are necessary effectors for 5T33 MM cell killing following *in vivo* IL-2 administration.

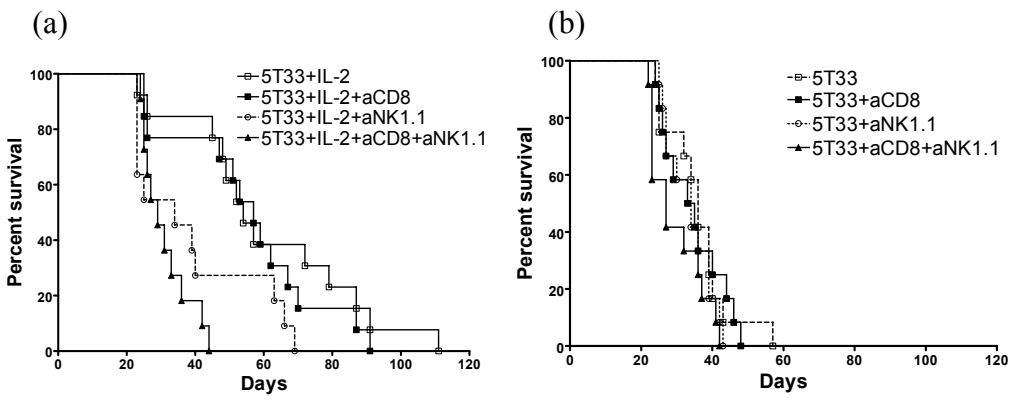


Figure 5: In vivo depletion of effector cells in 5T33 inoculated and IL-2 treated C57BL/KaLwRij mice. (a) IL-2 treated mice (b) untreated mice.

IL-2-activated NK cells from C57BL/KaLwRij mice showed marked cytotoxicity against syngeneic 5T33 tumor cells. In contrast, no significant cytotoxicity was detected against syngeneic LPS blasts, splenocytes, liver and normal BM cells.

Administration of IL-2-activated NK cells alone did not markedly, or only to a small extent, prolongs survival of MM bearing mice. However, when IL-2 activated NK cells were administered in conjunction with IL-2 treatment of mice, a significant prolongation of survival was observed. Survival of mice was significantly longer than observed with either treatment alone (Figure 6).

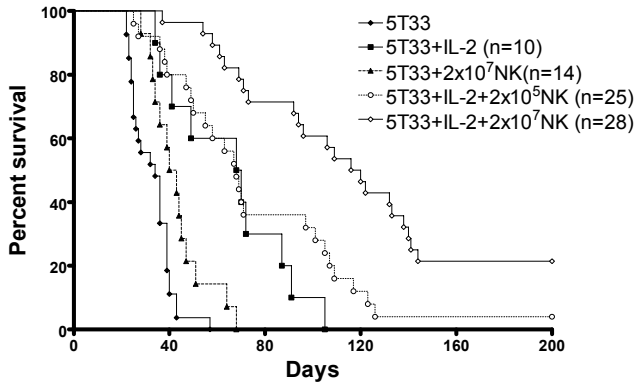


Figure 6: *In vivo* therapeutic effects of adoptively transferred IL-2 activated NK cells in IL-2 treated or control 5T33 inoculated C57BL/KaLwRij mice.

To follow the behaviour of adoptively transferred NK cells, they were retrovirally marked with the DsRed gene. Flow cytometry analysis of cells derived from the animals inoculated with DsRed$^+$ NK cells showed significant numbers of NK cells in the organs where MM cells resided. That is, the co-localization of NK and MM cells in the liver, spleen and BM suggested that adoptively transferred cells were homing to the tumor cell rich organs. By taking into account the total number of cells in these organs and the

corresponding percentage of the NK cell population, we deduced that the latter were proliferating at the tumor site.

ER targeted IL-2 confines autocrine growth stimulation to NK-92 cells

Due to the effects of IL-2 on different cell populations *in vivo*, and the side effects such as cytokine leak syndrome, we wanted to create a retroviral vector where NK cells are autoactivated by internal IL-2 production (paper IV). Consequently we cloned the IL-2 gene into a retroviral vector and targeted it to different subcellular compartments; the cytoplasm and the endoplasmic reticulum (ER). Thereafter, the IL-2 dependent NK-92 cell line was used to verify the functionality of these subcellularly targeted IL-2 constructs.

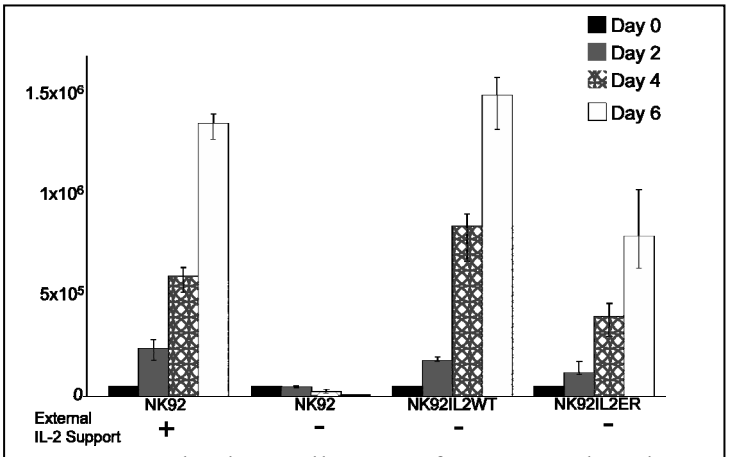


Figure 7: Absolute cell count of IL-2 transduced and control NK-92 cells over a 6-day culture period.

NK-92 cells transduced with the normal construct grew in the absence of exogenous IL-2.

Surprisingly, NK-92 cells transduced with the KDEL-tagged construct also grew in the absence of exogenous IL-2, and did so at a rate similar to that of nontransduced NK-92 cells grown with exogenous IL-2 (Figure 7). From the initial cultures, two new IL-2- independent NK-92 cell lines were established: one that secreted IL-2 (NK92IL2WT) and one that generated ER resident IL-2 (NK92IL2ER).

The growth characteristics of these cell lines, NK92IL2WT and NK92IL2ER, have been stable after continuous culture for almost a year. The cytotoxicity of gene modified cells against K562 were comparable to the nontransduced NK-92 cell line, suggesting no side effect of genetic manipulation in terms of NK cell activity.

In order to see the amount of IL-2 leakage in NK92IL2ER, these cells were then mixed in a 1:1 ratio with GFP-transduced NK-92 cells (NK92GFP) and co-cultured in the absence of exogenous IL-2. In these experiments, NK92IL2ER cells did not support the growth of the GFP-transduced NK-92 cells, indicating no or only very low levels of released IL-2. In contrast, NK92IL2WT cells readily supported growth of bystander GFP-transduced cells under similar conditions. This result suggests that ER retained expression of IL-2 is sufficient for autocrine growth stimulation to NK cells while not secreting IL-2 to the extracellular compartment.

Discussion

Multiple Myeloma is an incurable plasma-cell neoplasm with a median length of survival after diagnosis of approximately three years. Although the disease remains incurable, current main alternatives are drug treatment modalities[46] and autologous stem cell transplantation (ASCT). Gene marking can be used to investigate if progenitor cells harvested from patients during ASCT are contaminated with tumorigenic cells. It can also provide information about the contribution of hematopoietic stem cells to long-term engraftment and about long term transgene expression from integrated retroviral vectors. In order to study autologous infused cell contribution to relapse as well as the long term persistence of the transgene in hematopoietic cells following ASCT for multiple myeloma, we genetically marked autologous $CD34^+$ enriched bone marrow or peripheral blood cell grafts of eight myeloma patients using retroviral vectors. Six patients were subsequently transplanted with the marked graft and followed with regular time points of analysis. Briefly, mononuclear cells were harvested by leukapheresis during 2-4 consecutive days following priming with GM-CSF or G-CSF. The $CD34^+$ cells separated on Cellpro ceprate avidin-biotin columns were exposed to the G1Na vector coding for neomycin resistance gene at a ratio of 5 vector particles per cell at three consecutive time points achieving an average transduction efficacy of 2% (0.43% -5.1%). The patients were transplanted with a mixture of transduced cells and un-manipulated graft. Vector integration and transgene expression were analyzed by colony assays and PCR. The transgene could be detected for up to five years post-transplant in normal bone marrow cells, even in remission following relapse and no side effects related to retroviral gene transfer were observed. There were no marked myeloma cells observed in the patients either in remission or in relapsing disease which indicates that contribution of infused cells to relapse is unlikely. These findings, together with other studies, support the idea that the lack of complete eradication of residual myeloma cells by conditioning rather than the re-introduced myeloma cells during ASCT is the cause of relapse and therefore, new treatment modalities to eradicate the disease are needed.

Based on these findings, we decided to return to experimental models where we can set up a model to analyse novel myeloma treatment modalities. Among several

myeloma models, the spontaneous development of MM in C57BL/KaLwRij mice has several advantages, mainly it resembles the human disease. Murine MM arises spontaneously in aged C57BL/KaLwRij mice at a frequency of about 1%. However, when established autologous myeloma cell lines (5T33 and 5T2) are inoculated intravenously into the syngeneic young mice, all of the inoculated mice develop MM after a short time (4-5 weeks). In this study, 5T33MM cells were transduced with a retroviral vector coding for GFP. Genetically marking the myeloma cells facilitated the analysis of kinetics and tissue distribution of these cells after in vivo administration and thus enabled us to follow and characterise both the course of the disease and the effect of novel treatment modalities. The GFP-marked myeloma cells were sorted by FACS to reach about 100% purity and then were injected into immunocompetent C57BL/KaLwRij mice. The inoculated mice were monitored for the development of MM. At the time of disease development, the mice were killed, organs were removed and analysed for the presence of GFP-marked myeloma cells using FACS. eGFP detection was confirmed to be accurate at 1:1000 transduced/non-transduced cell ratio. eGFP-5T33 and 5T33 cells exhibited similar growth rates, and produced comparable IgG2b and interleukin-6 levels. In vivo challenge of both cells resulted in a similar disease development. eGFP-transduced MM cells were detected substantially in the bone marrow, spleen, liver, less in lymph nodes, but not in the thymus. The bone marrow of paraplegic mice contained higher eGFP-transduced MM cells compared to that of non-paraplegic animals. The establishment of this model not only simplified the analysis of homing pattern studies, but also eased the evaluation of therapeutic effects of different treatment approaches.

Effector cells in peripheral blood against mouse autologous multiple myeloma target cells have, in previous reports, shown limited cytotoxicity that could be increased after IL-2 activation. Therefore, using this mouse model, we aimed to determine if NK cells provide anti-MM activity following IL-2 administration, and if ex vivo activated and intravenously (i.v.) administered NK cell prolong survival in MM bearing C57BL/KaLwRij mice. For MM induction, C57BL/KaLwRij mice were inoculated i.v. with syngeneic 5T33 MM cells. The effect of IL-2 was tested by intraperitoneal injection into the 5T33 tumor inoculated mice. In vivo effector cell depletions were performed by administration of anti-NK1.1 or anti-CD8 monoclonal antibodies. In vitro NK cell cytotoxicity assays were performed against 5T33 MM

cells using a ^{51}Cr-release assay. Magnetically separated and IL-2 activated NK cells from splenocytes of C57BL/KaLwRij mice were used for cytotoxicity assays and for adoptive transfers. NK cells were also genetically marked before adoptive transfer for assessment of biodistribution and localization to areas of tumor growth. IL-2 administration into MM bearing mice significantly prolonged their survival. The effects of IL-2 were diminished by in vivo depletion of NK cells. Adoptively transferred IL-2 activated NK cells in conjunction with IL-2 treatment showed a significant anti-MM effect in vivo. The effect was greater than administration of either IL-2 or adoptively transferred NK cells alone. Biodistribution of the marked adoptively transferred NK cells correlated with MM cells' homing sites. These data strongly support that NK cells are not only the main effectors responsible for autologous myeloma cell killing in the C57BL/KaLwRij myeloma model, but also they increase its life expectancy through adoptive transfer.

Due to the effects of IL-2 on different cell populations in vivo, and the side effects such as cytokine leak syndrome in humans, we wanted to create a retroviral vector where NK cells are autoactivated by internal IL-2 production. Consequently we cloned the IL-2 gene into a retroviral vector along with retention signals for different subcellular compartments; the cytoplasm and the ER. Thereafter, the IL-2 dependent NK-92 cell line was used to verify the functionality of these subcellularly targeted IL-2 constructs. IL-2-transduced NK-92 cell lines showed comparable functional activity and cytotoxicity to parental NK-92 cells and excelled as a promising tool for adoptive immunotherapy. The ER targeted IL-2 expression in NK92IL2ER was verified to be localised and restricted in the ER. IL-2 was not detected in the supernatant and non-IL-2-modified NK-92 cells were not supported during co-culturing experiments.

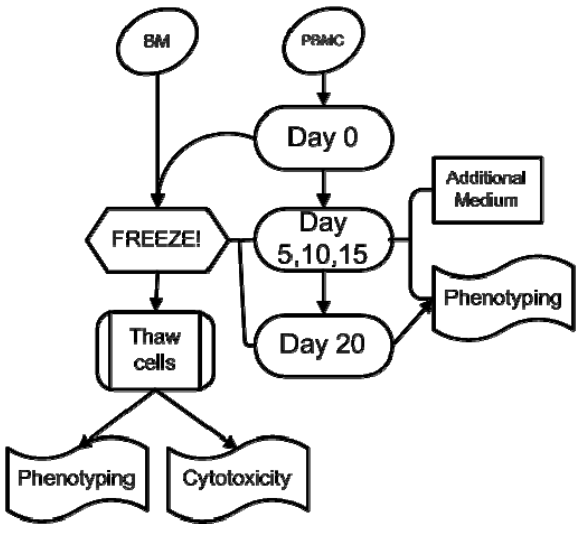
The above findings suggest that natural killer cells are one of the important effector cells against multiple myeloma and IL-2 is an important factor for their anti-myeloma activity. This indicates that IL-2 induced NK cells (gene modified or unmodified) can be analysed for feasibility in human settings.

Ongoing research

As shown in paper IV, although there is anti-myeloma activity of NK cells coupled with IL-2, the effect can still be enhanced. Thus, an obvious future perspective of this project could be to transduce NK cells derived from mice, with the above mentioned human IL-2 constructs, which should then be tested for their efficiency in a mouse setting, or to use similar murine IL-2 constructs. This way it will be possible to compare the effects of exogenously IL-2-activated NK cells with the IL-2ER transduced NK cells against MM.

Since our data suggest that NK cells might have a therapeutic potential in MM especially when induced by IL-2, we are trying to investigate the feasibility of expanding cytokine induced NK (CINK) cells from peripheral blood of MM patients with the aim of reaching clinically relevant doses for use particularly in association with autologous transplantation as a supportive or preemptive therapy for MM. For this purpose, NK cells from PBMCs of 7 patients with MM were expanded according to our previously optimized protocol[359] and their cytotoxic activity against autologous myeloma cells was evaluated.

Figure 8: Expansion protocol



In short, PBMCs from MM patients were initially cultured at a concentration of $0.5x10^6$ cells/ml [360] in CellGro SCGM serum-free medium with the addition of 5% human serum and 500 U/ml rhIL-2. For the first 5 days, the medium was further supplemented with anti-CD3 antibody to a final concentration of 10 ng/ml. On days 5–6, the OKT-3-containing medium was washed out, and fresh medium with IL-2 (500 U/ml) but without OKT-3 was added. The cultures were replenished with fresh medium every 2–3 days throughout the culture period. During the 20 day culture period, cells were followed up for total cell numbers and the corresponding percentages of NK, NK-like T and T

cells were determined by flow cytometry. Moreover, in the beginning (Day 0) and end (Day 20) of culture, cells were subjected to a detailed phenotyping panel including flourochrome conjugated MAbs against several different surface antigens in order to track any phenotypic differences that might occur during this period.

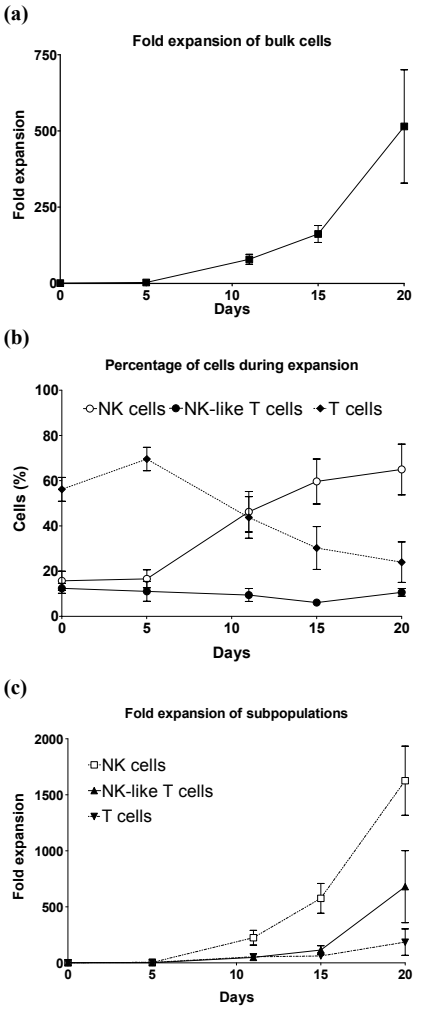


Figure 9: Expansion dynamics of samples obtained from seven myeloma patients and cultured for 21 days under identical conditions. Plotted data is mean±SD. (a) Fold expansion of bulk cells in culture. The mean expansion was 511 fold. (b) Dynamics of subpopulations as percentages of total cell counts. Initial OKT-3 treatment lead to increased percentage of T cells in the culture during the first 5 days which was followed by a decrease after withdrawal of OKT-3. This lead to subsequent increase and finally domination of NK cells in the culture. (c) Fold expansion of subpopulations. Culture conditions favored the expansion of NK cells whereas NK-like T cells were expanded at a lower rate followed by T cells at an even lower rate.

We have observed that NK, T and B cell counts at the start of the culture (Day 0) in different patients were variable and the mean percentage of NK cells was 10,57% (range: 5-15%). After 21 days of culture, all PBMC samples showed a dramatic expansion of $CD56^{+}$ $CD3^{-}$ cells. The expansion approached log-linearity after an initial "resting" phase after 5 days. A high rate of bulk cell expansion as well as a significant increase in the percentage of NK cells in culture was observed (Figure 9a, Figure 9b). The relatively higher expansion rate of NK cells shows that our culture conditions support the growth of NK cells compared to other types of cells in the culture environment. At day 20, a mean of 511-fold (range: 123-1545) bulk cell expansion and an NK cell content of 64% (range: 7-95%) was observed. There was a significant increase in absolute number of NK cells and the final products had a mean of 1625-fold (range: 502-2658) NK cell expansion (Figure 9c). The expansion reaches a plateau level after 21 days culture period and the expression levels of CD2 and CD7, which are both highly expressed in NK cell lymphomas/leukemias[361,362], were not altered during expansion suggesting that there is no malignant transformation of the cells during expansion.

Phenotypic analysis of these cells before and after expansion revealed a significant upregulation in the expression of 10 activating receptors including 2B4, CD8, CD16, CD27, CD226, NKG2C, NKG2D, NKp30, NKp44 and NKp46 whereas the inhibitory receptors KIR2DL3 and Lir-1 were also upregulated. The chemokine receptor CCR7 was significantly downregulated while CXCR3 was drastically upregulated. We observed no significant changes in the expression levels of CD2, CD7, CD57, CD62L, CD69, CD161, LFA-1, GL183, KIR2DL1, NKB1, NKG2A, CCR5 or CXCR4 (Figure 10).

Due to the fact that MM cells have normal levels of CD48 (the Ligand for 2B4), the impairment of 2B4 expression in NK cells from MM patients[292] might be an important factor playing role in the immune escape mechanism of MM, therefore its upregulation after *ex vivo* expansion is most likely one of the major factors contributing to the observed increase in cytotoxicity against autologous MM cells. Besides, NCRs and NKG2D, which are previously shown to take part in the recognition of MM cells by NK cells[291], are significantly upregulated, suggesting possible pathways for autologous myeloma cell killing. Also of importance is the upregulation of CD226, which is thought to function as a tumor surveillance receptor in NK cells[363], and a potent inducer of cytotoxicity against many tumor cell lines of hematopoetic and non-hematopoetic origin[235]. Thus, its upregulation in expanded CINKs could also have a significant contribution to the increase in cytotoxicity.

It has previously been shown that CD27^{low} NK cells are tightly regulated by inhibitory receptors whereas CD27^{high} NK cell subset displays a greater effector function[364]. Due to the increase in CD27 during our expansion protocol, the upregulation of inhibitory

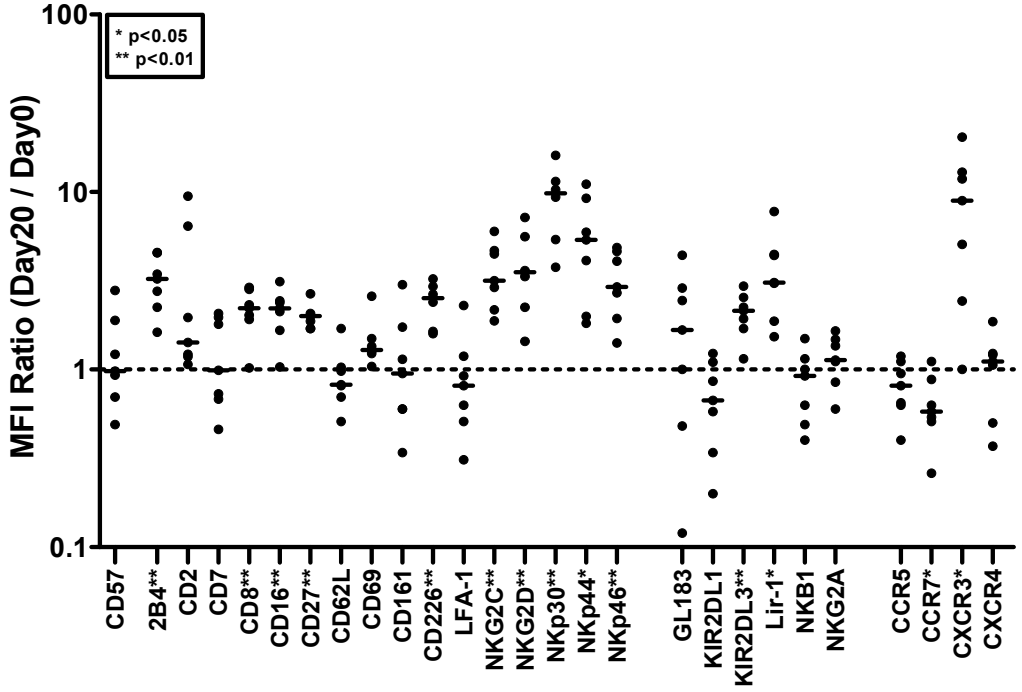


Figure 10: Changes in the receptor expression patterns of NK cells following expansion and activation. Each patient's mean fluorescence intensity ratios (MFIR) (•) of Day 20 to Day 0 as well as the medians (—) of seven patients were plotted for each receptor. The dashed line shows MFIR=1 which indicates unaltered receptor expression during the expansion period. Above the line (MFIR>1) are upregulated values and below (MFIR<1) are downregulated.

receptors KIR2DL3 and Lir-1 might be insufficient to suppress the activating signals. The same report also demonstrates that BM NK cells mainly consist of CD27^{high} cells, which suggests that the final expansion products that have a high expression of CD27 are more likely to localize in the BM. Furthermore, the upregulated chemokine receptor CXCR3 is shown[365] to mobilize NK cells from spleen but not from BM and drive their migration towards sites of inflammation. Taken together, these results indicate that expanded CINK cells have an increased likelihood of co-localizing with MM cells in the BM thus creating optimal conditions for effective cytotoxicity *in vivo*.

To evaluate the potential use of this procedure in MM therapy the cytotoxic capacity of NK cells before and after expansion were measured *in vitro* by a standard 4 hour ^{51}Cr-release assay against NK-sensitive K562 cells and by a flow cytometry based

cell-mediated cytotoxicity assay against autologous MM cells. Cytotoxicity against K562 showed a marked increase in cytotoxic activity of day 20 NK cells when compared to day 0 (Figure 11a). At 10:1 NK to myeloma cell ratio, expanded CINK cells killed 61.69 % of K562 targets whereas day 0 NK cells showed only baseline levels of cytotoxicity. At day 20, cells showed a significantly increased cytotoxic activity against autologous myeloma cells whereas day 0 cells, again, showed only baseline levels of cytotoxicity (Figure 11b). At 10:1 effector:target ratio, 61.41% of autologous myeloma cells were killed. Moreover, there was no significant cytotoxicity against autologous $CD138^-$ $CD38^-$ cells. This shows that expanded CINK cells show a strong and highly specific cytotoxic activity against autologous myeloma cells *in vitro*.

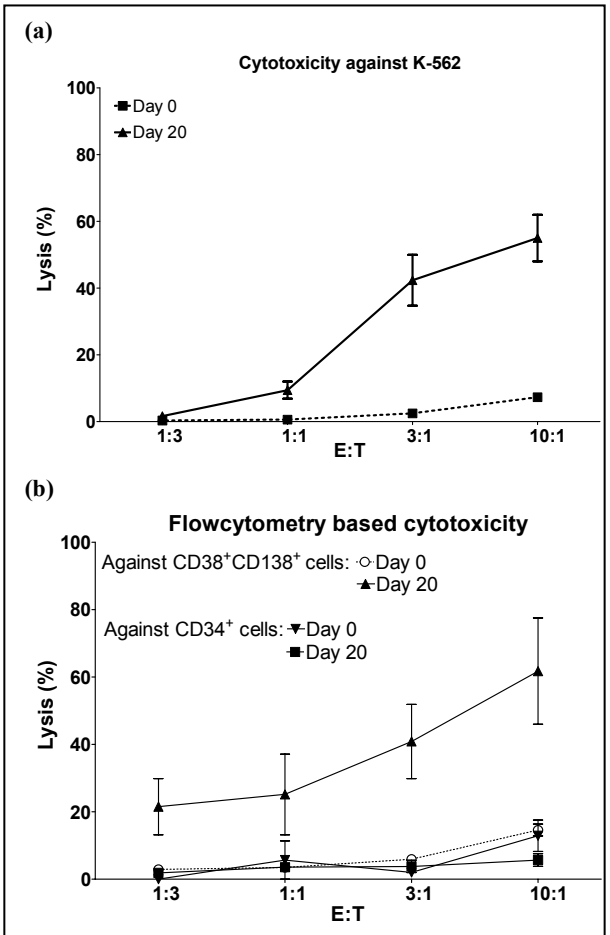


Figure 11: Cell mediated cytotoxicity assays. (a) NK cell cytotoxicity against K562 cell line as measured by standard chromium release assay. **(b)** Flowcytometry based cytotoxicity assay demonstrating an increased cytotoxicity of expanded CINK cells (Day 20) against autologous myeloma cells compared to freshly isolated NK cells (Day 0). Expansion and activation of the cells did not affect the cytotoxicity against autologous normal marrow cells such as $CD34^+$ cells.

To our knowledge, this is the first study to demonstrate that NK cells from MM patients can be efficiently expanded *ex vivo* and that these cells show a significantly higher cytotoxic activity against autologous primary myeloma cells than freshly isolated NK cells while not killing normal cells. Moreover, the use of GMP quality components in the procedure ensures the feasibility of this application for clinical use.

In conclusion, we have clearly shown that *ex vivo* expanded CINK cells show high levels of autologous anti-myeloma activity *in vitro*. Moreover, we observed no significant cytotoxicity against autologous normal cells. Using the *ex vivo* expansion protocol presented here, it now seems feasible to reach desired treatment dose and percentage of NK cells from MM

patients with GMP quality components. These results suggest that expanded CINK cells selectively kill autologous myeloma cells and they can be used as a tool to treat the disease.

Acknowledgments

"1916, Northampton, England

When the war finally came to an end, I was at a loss as to what to do... I took stock of my qualifications. A not-very-good degree, redeemed somewhat by my achievements at the Admiralty. Knowledge of certain restricted parts of magnetism and hydrodynamics, neither of them subjects for which I felt the least bit of enthusiasm. No published papers at all... Only gradually did I realize that this lack of qualification could be an advantage. By the time most scientists have reached age thirty they are trapped by their own expertise. They have invested so much effort in one particular field that it is often extremely difficult, at that time in their careers, to make a radical change. I, on the other hand, knew nothing, except for a basic training in somewhat old-fashioned physics and mathematics and an ability to turn my hand to new things... Since I essentially knew nothing, I had an almost completely free choice..."

Francis Crick,

What Mad Pursuit, Basic Books, New York, 1988, pp 15-16.

I felt exactly like Prof. Crick expressed above when I first came to Sweden. I had no knowledge about gene therapy, cell therapy or tumor immunology. I am extremely lucky to then have met a person that changed my life: Associate Professor **Sirac Dilber,** *my supervisor*. I spent time with Sirac almost every evening until late nights reading and discussing about science during the first two years. He, together with Alar and Manuchehr, literally thought me everything I know about gene therapy and immunotherapy. When I think about you, Napoleon Bonaparte's famous quote comes to my mind: "Impossible is a word to be found only in the dictionary of fools." Thank you, Sirac.

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